

1 Anti-CD3 Immunotoxins  
2 and Therapeutic Uses Therefor  
3

4 Field of the Invention  
5

6 The present invention relates to recombinant immunotoxins  
7 comprising a CD3-binding domain and a *Pseudomonas* exotoxin A  
8 mutant.  
9

10 Background of the Invention  
11

12 On the surface of every mature T cell are T-cell receptor  
13 (TCR) molecules consisting of a heterodimer of polypeptide  
14 chains  $\alpha$  and  $\beta$  (or alternatively, chains  $\gamma$  and  $\delta$ ). The TCR  $\alpha:\beta$   
15 heterodimers, of which there are some 30,000 on every cell, are  
16 capable of engaging with the major histo-compatibility complex  
17 (MHC) on an antigen-presenting cell (APC), and thereby account  
18 for antigen recognition by all functional classes of T cells.  
19 The  $\alpha:\beta$  heterodimer itself does not appear to be involved in  
20 signal transduction following TCR engagement by specific MHC-  
21 peptide antigen complexes. Rather, that function is provided by  
22 a complex of proteins which is stably associated with the TCR  $\alpha\beta$   
23 or  $\gamma\delta$  heterodimers on the surface of all peripheral T-cells and  
24 mature thymocytes, namely, the CD3 complex. The human CD3  
25 complex comprises six polypeptides with usually four different  
26 chains:  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ . Three different dimers constitute the  
27 CD3 complex ( $\gamma\epsilon$ ,  $\delta\epsilon$ , and  $\zeta\zeta$ ), Leukocyte Typing VI, ed. by  
28 Kishimoto et al., Garland Publishing, Inc., 1998, p. 44. The  
29 CD3 proteins are absolutely essential for cell-surface  
30 expression of the T-cell receptor chains. Mutants lacking  
31 either of the TCR chains or any of the  $\gamma$ ,  $\delta$  or  $\epsilon$  chains of the

1 CD3 complex, fail to express any of the chains of the TCR at the  
2 cell surface. See Janeway, C.A., Jr. and P. Travers,  
3 Immunobiology. The Immune System in Health and Disease, Ch. 4  
4 ("Antigen Recognition by T Lymphocytes"), Current Biology Ltd.,  
5 London and Garland Publishing Inc., New York, 1996.  
6

7 Antigen-specific T cell activation and clonal expansion  
8 occur when two signals are delivered by APC to the surface of  
9 resting T lymphocytes. The first signal, which confers  
10 specificity to the immune response, is mediated via the TCR  
11 following recognition of foreign antigenic peptide presented in  
12 the context of MHC. Optimal signaling through the TCR requires  
13 a clustering of the TCR with co-receptors CD4 or CD8. This in  
14 turn results in increased association of cytosolic tyrosine  
15 kinases with the TCR and the CD3 cytoplasmic tails, as well as  
16 with CD45. Phosphorylation of the cytoplasmic domain of CD3 $\epsilon$  and  
17  $\zeta$  results in binding of tyrosine kinases, initiating a series  
18 of intracellular events resulting in the proliferation and  
19 differentiation of the T cell. The second signal, termed  
20 "costimulation," which is neither antigen-specific nor MHC  
21 restricted, is provided by one or more distinct cell surface  
22 molecules expressed by APC's. Janeway and Travers, *supra* at 4-  
23 28.  
24

25 Delivery of an antigen-specific signal with a costimulatory  
26 signal to a T cell leads to T cell activation, which can include  
27 both T cell proliferation and cytokine secretion. The  
28 combination of antigen and co-stimulator induces naïve T cells  
29 to express IL-2 and its receptor. IL-2 induces clonal expansion  
30 of the naïve T cell and the differentiation of its progeny into  
31 armed effector T cells that are able to synthesize all the

1 proteins required for their specialized functions as helper,  
2 inflammatory, and cytotoxic T cells, see, e.g., Janeway and  
3 Travers, *supra* at §§7-8, 7-9.

4  
5 The adaptive immune mechanisms described above constitute a  
6 major impediment to successful organ transplantation. When  
7 tissues containing nucleated cells are transplanted from a donor  
8 to a graft recipient, T-cell responses in the recipient to the  
9 typically highly polymorphic MHC molecules of the graft almost  
10 always trigger an immediate T-cell mediated response against the  
11 grafted organ. The use of potent immunosuppressives such as  
12 cyclosporin A and FK-506 to inhibit T cell activation has  
13 increased graft survival rates dramatically, but with certain  
14 disadvantages, including life-long dependence on the drug by the  
15 graft recipient.

16  
17 Development of improved means of immunosuppression in  
18 patients receiving organ transplants, or suffering from T-cell  
19 mediated immune disease, has been a constant objective in the  
20 field of transplantation. A particular objective of workers in  
21 the art is development of a therapeutic agent capable of  
22 inducing donor-specific immunologic tolerance in a patient, and  
23 thereby freeing the patient from otherwise continuous dependence  
24 on immunosuppressives.

25  
26 The term "immunological tolerance" refers to a state of  
27 unresponsiveness by the immune system of a patient subject to  
28 challenge with the antigen to which tolerance has been induced.  
29 In the transplant setting, in particular, it refers to the  
30 inhibition of the graft recipient's ability to mount an immune  
31 response which would otherwise occur in response to the  
32 introduction of non-self MHC antigen of the graft into the

1 recipient. Induction of immunological tolerance can involve  
2 humoral, cellular, or both humoral and cellular mechanisms.  
3

4       Systemic donor-specific immunological tolerance has been  
5 demonstrated in animal models as well as in humans through  
6 chimerism as a result of conditioning of the patient through  
7 total body irradiation or total lymphoid irradiation, prior to  
8 bone marrow transplantation with donor cells, Nikolic, B. and  
9 Sykes, M. (1997) *Immunol. Res.* 16: 217-228.  
10

11       However, there remains a critical need for a conditioning  
12 regimen for allogeneic bone marrow transplantation that will  
13 result in stable mixed multilineage allogeneic chimerism and  
14 long-term donor-specific tolerance, in the absence of radiation.  
15 Hematologic abnormalities including thalassemia and sickle cell  
16 disease, autoimmune states, and several types of enzyme  
17 deficiency states, have previously been excluded from bone marrow  
18 transplantation strategies because of morbidity associated with  
19 conditioning to achieve fully allogeneic bone marrow  
20 reconstitution. Conditioning approaches which do not involve  
21 radiation may significantly expand the application of bone marrow  
22 transplantation for non-malignant diseases.  
23

24       Immunotoxins comprising an antibody linked to a toxin have  
25 been proposed for the prophylaxis and/or treatment of organ  
26 transplant rejection and induction of immunological tolerance.  
27 For example, a chemically conjugated diphtheria immunotoxin  
28 directed against rhesus CD3 $\epsilon$ , i.e. FN18-DT390, has been used in  
29 primate models of allograft tolerance and also in primate islet  
30 concordant xenograft models, see Knechtle et al. (1997)  
31 *Transplantation* 63:1, Neville et al. (1996) *J. Immunother.* 19:  
32 85; Thomas et al. (1997) *Transplantation* 64: 124; Contreras et

1 al. (1998) *Transplantation* 65: 1159-1169. Additionally, a  
 2 chemically coupled *Pseudomonas* immunotoxin, LMB-1 B3(Lys)-PE38,  
 3 has been used in clinical trials against advanced solid tumors,  
 4 Pai, L.H. and I. Pastan, *Curr. Top. Microbiol. Immunol.* 234:83-96  
 5 (1998). However, product heterogeneity is a significant  
 6 practical difficulty associated with chemically conjugated  
 7 immunotoxins.

8  
 9 A single chain recombinant immunotoxin comprising the  
 10 variable region of an anti-CD3 antibody, UCHT-1 and a diphtheria  
 11 toxin, has been proposed as a therapeutic agent, see WO 96/32137,  
 12 WO 98/39363. However, early vaccination of the general  
 13 population against diphtheria raises concerns about pre-existing  
 14 antibodies to the toxin in many patients. Alternately, a  
 15 recombinant immunotoxin comprising anti-Tac linked to PE38 is  
 16 also proposed as a prophylaxis and treatment against organ  
 17 transplantation and autoimmune disease, see Mavroudis et al.  
 18 (1996). *Bone Marrow Transplant.* 17: 793.

19  
 20 It has been an object to achieve a recombinant immunotoxin  
 21 having directed toxic effect at high levels against T cells,  
 22 which thereby provides improvements in the prophylaxis or  
 23 treatment of transplant rejection and in the induction of  
 24 immunologic tolerance, as well as in the treatment or prevention  
 25 of graft versus host disease (GVHD), autoimmune disease, and  
 26 other T-cell mediated diseases or conditions.

27  
 28 It has also been an object to provide an immunotoxin  
 29 against which the recipient is normally free of pre-existing  
 30 antibodies.

31

1       We have now discovered that recombinant fusions of a CD3-  
2   binding domain and a *Pseudomonas* exotoxin A mutant provide an  
3   immunotoxin having potent anti-T cell effect. The immunotoxins  
4   of the invention provide improvements in the clinical treatment  
5   or prevention of transplant rejection, graft-versus-host disease  
6   (GVHD), T-cell mediated autoimmune disease, T-cell leukemias, or  
7   lymphomas which carry the CD3 epitope, acquired immune  
8   deficiency syndrome (AIDS), and other T-cell mediated diseases  
9   and conditions.

10  
11  
12   Summary of the Invention

13  
14       The present invention is directed to isolated recombinant  
15   immunotoxins comprising a CD3-binding domain and a *Pseudomonas*  
16   exotoxin A component, and pharmaceutically acceptable salts  
17   thereof; to in vivo and ex vivo methods for the treatment and  
18   prophylaxis of organ transplantation rejection and graft-versus-  
19   host disease, and for the induction of immunologic tolerance, as  
20   well as for treatment or prophylaxis of auto-immune diseases,  
21   AIDS and other T-cell mediated immunological disorders, and T-  
22   cell leukemias or lymphomas, using the immunotoxins or  
23   pharmaceutically acceptable salts thereof; and to pharmaceutical  
24   compositions comprising the novel immunotoxins or their  
25   pharmaceutically acceptable salts.

26  
27       The invention also concerns polynucleotides and  
28   physiologically functional equivalent polypeptides which are  
29   intermediates in the preparation of the subject recombinant  
30   immunotoxins; recombinant expression vectors comprising said  
31   polynucleotides, procaryotic and eucaryotic expression systems,  
32   and processes for synthesizing the immunotoxins using said

1 expression systems; and methods for purification of the  
2 immunotoxins of the invention.

3  
4 In particular, the invention relates to a novel recombinant  
5 immunotoxin, scFv(UCHT-1)-PE38, which is a single chain ("sc")  
6 Fv fragment of murine anti-human CD3 monoclonal antibody, UCHT-  
7 1, fused to a truncated fragment of *Pseudomonas aeruginosa*  
8 exotoxin A, i.e. PE38. For example, we have found said  
9 scFv(UCHT-1)-PE38 to be highly effective in T-cell killing in  
10 vitro; and we have further found that the immunotoxin is capable  
11 of ablating murine CD3/human CD3 double positive T cells at high  
12 levels in a dose-dependent manner in vivo in mice transgenic for  
13 human CD3ε.

14  
15  
16 Brief Description of the Figures.

17 FIG. 1 Schematic diagram showing domain organization of  
18 scFv(UCHT-1)-PE38 molecule prepared in Example  
19 1, consisting of an N-terminal light chain  
20 variable region (V<sub>L</sub>) of 109 residues, a peptide  
21 linker (L) of 16 residues, a heavy chain  
22 variable region (V<sub>H</sub>) of 122 amino acids, a  
23 connector segment (C) of 5 amino acids (KASGG)  
24 (SEQ. ID. NO:9) , and the PE38 mutant,  
25 comprising 347 amino acids ("Toxin").  
26

27 FIG. 2 Schematic map of pET15b expression plasmid  
28 prepared in Example 1 for expression of  
29 scFv(UCHT-1)-PE38 expression under control of  
30 bacteriophage T7 promoter (pT7) in E. coli.  
31 Relevant restriction sites, i.e. Nco I, Hind III  
32 and Bam HI/Bgl II, are noted. The peptide  
33 linker, (Gly<sub>3</sub>Ser)<sub>4</sub> (SEQ. ID. NO: 5), is shown

- 1 linking the carboxy terminus of  $V_L$  to the amino
- 2 terminus of  $V_H$ .
- 3
- 4 FIG. 3 Typical elution profiles from anion-exchange
- 5 columns used to purify scFv(UCHT-1)-PE38 in
- 6 Example 1. (A) Step elution from Fast-Flow Q
- 7 (Pharmacia). (B) Salt gradient elution from Q5
- 8 (BioRad).
- 9
- 10 FIG. 4 SDS-PAGE gel of scFv(UCHT-1)-PE38 (Lane 1: High
- 11 molecular weight markers (Amersham); Lane 2: 2  $\mu$
- 12 g refolded and concentrated protein prior to
- 13 anion exchange column chromatography; Lane 3: 2
- 14  $\mu$ g protein eluting at the peak position of the
- 15 Fast Flow Q column ; Lane 4: 2  $\mu$ g protein eluting
- 16 at the peak position from the Q5 column; Lane 5:
- 17 High molecular weight markers (Amersham),
- 18 including bovine serum albumin at 66 kD).
- 19
- 20 FIGS. 5A,B (A) Absorbance profile at OD<sub>260</sub> of scFv(UCHT-1)-
- 21 PE38 on size exclusion chromatography (Sephacryl
- 22 S200). (B) Mobility relative to the mobility of
- 23 marker proteins ( $\beta$ -amylase, 200 kD; alcohol
- 24 dehydrogenase, 150 kD; bovine serum albumin, 66
- 25 kD; carbonic anhydrase, 29 kD; cytochrome c, 12.4
- 26 kD).
- 27
- 28 FIG. 6 Protein synthesis in Jurkat (CD3<sup>+</sup>) compared to
- 29 Ramos (CD3<sup>-</sup>) cells treated with increasing molar
- 30 concentrations of scFv(UCHT-1)-PE38 (Pooled
- 31 batches 12-16 and 10A-12A of Example 1), as a



1 percent of protein synthesis in control, untreated  
2 cells of the respective type.

3  
4 FIGS. 7A,B Inhibition of human mixed leukocyte reaction by  
5 scFv(UCHT-1)-PE38 or cyclosporine A (CsA)  
6 (positive control). As reported in Example 1, two  
7 different experiments, graphically represented in  
8 7A and 7B, utilize cells from three different  
9 donors (A, B and C) in combinations A<->B, A<->C  
10 and B<->C. <sup>3</sup>H-TdR uptake by treated cells  
11 (relative to control, non-treated cells) is  
12 plotted against immunotoxin concentration (ng/ml)  
13 or CsA concentration (nM).  
14

15 FIG. 8 Comparison of the effect of scFv(UCHT-1)-PE38 on  
16 proliferation of Con A-stimulated splenocytes  
17 from transgenic mice ("HuCD3εTg cells") vs. cells  
18 from non-transgenic, B6CBAF1 mice ("NonTg  
19 cells"). <sup>3</sup>H-thymidine incorporation (in counts per  
20 million, CPM) by the Concanavalin A ("ConA")-  
21 stimulated T cells is plotted against scFv(UCHT-  
22 1)-PE38 concentration (ng/ml). Values represent  
23 the average of triplicate samples, and error bars  
24 represent the standard deviation. Solid  
25 horizontal lines represent the proliferative  
26 response in the absence of ConA, i.e. due to  
27 media alone: for the transgenic cells, this  
28 value is 342 cpm; for the nontransgenic cells  
29 this value is 112 cpm (not shown). In the  
30 transgenic cells, the value for a 50%  
31 proliferative response is 11,101 cpm. As reported  
32 in Example 1, the immunotoxin blocks ConA-

1 induced proliferation of HuCD3 $\epsilon$ Tg cells on a  
2 dose-dependent basis, but not of NonTg cells.  
3

4 FIGS. 9A,B <sup>3</sup>H-Thymidine incorporation (CPM) in one-way MLR.  
5 scFv(UCHT-1)PE38 (ng/ml) is shown to inhibit  
6 mitomycin C-induced proliferation of transgenic  
7 murine T cells expressing human CD3 $\epsilon$  cells ("CD3Tg  
8 cells") but not of non-transgenic, B6CBAF1  
9 splenocytes ("NonTg cells"). Values represent the  
10 average of triplicate samples, and error bars  
11 represent the standard deviation. The line  
12 labelled "No stimulator cells" represents the  
13 proliferative response in the absence of Balb/C  
14 splenocytes, due to media alone (FIG. 9A: 1651  
15 cpm; FIG. 9B: 342 cpm). In the transgenic cells,  
16 the value for a 50% proliferative response is  
17 3891 cpm (FIG. 9A) or 688 cpm (FIG. 9B).  
18

19 FIG. 10 Relative cell growth of CD3<sup>+</sup> Jurkat cells, as  
20 compared to CD3<sup>-</sup> LS174T and MDA-MB-435S cells, in  
21 hollow fibers implanted in the peritoneal cavity  
22 in nude mice (6 per group) administered  
23 scFv(UCHT-1)-PE38 by intraperitoneal injection (1  
24  $\mu$ g/mouse or 5  $\mu$ g/mouse). Controls taken at Day 0  
25 and on injection of vehicle alone are shown.  
26 Viable cell population is determined by MTS  
27 assay.  
28

29 FIGS. 11A,B,C Two-color FACS analysis of spleen cells from  
30 heterozygous tg $\epsilon$ 600 transgenic mice with and  
31 without scFv(UCHT-1)-PE38 treatment. A. Non-  
32 specific double staining of spleen cells from

1 untreated animals with isotype-matched control  
2 antibodies ("PE-Isotype" and "FITC-Isotype"). B.  
3 Double staining of spleen cells from untreated  
4 control animal with anti-mouse CD3-PE38 and anti-  
5 human CD3-FITC. C. Double staining with anti-  
6 mouse CD3-PE and anti-human CD3-FITC of spleen  
7 cells from an animal systemically treated with  
8 scFv(UCHT-1)-PE38 by intravenous injection.  
9

10 FIGS. 12A,B,C Two-color FACS analysis of lymph node (LN) cells  
11 from heterozygous tg $\epsilon$ 600 transgenic mice with and  
12 without scFv(UCHT-1)-PE38 treatment. A. Double  
13 staining of LN cells from untreated animals with  
14 isotype control antibodies (PE-Isotype and FITC-  
15 Isotype). B. Double staining of lymph node cells  
16 from an untreated control animal with anti-mouse  
17 CD3-PE and anti-human CD3-FITC. C. Double  
18 staining with anti-mouse CD3-PE and anti-human  
19 CD3-FITC of LN cells from an animal systemically  
20 treated with scFv(UCHT-1)-PE38 by intravenous  
21 injection.  
22

23 FIGS. 13A,B Decreasing fraction (A) and number (B) of  
24 transgenic human CD3-positive T spleen cells after  
25 systemic administration of scFv(UCHT-1)-PE38. The  
26 number of huCD3<sup>+</sup> cells is determined by  
27 multiplying the total number of cells recovered  
28 from the spleen by the fraction of total cells  
29 (shown in Fig. 12A) that are huCD3<sup>+</sup>. (p < 0.05 vs.  
30 untreated using one-way ANOVA of ranks).  
31

1 FIGS. 14A,B

2 Decreasing percentage (A) and number (B) of  
3 transgenic human CD3-positive lymph node (LN)  
4 cells after systemic administration of scFv(UCHT-  
5 1)-PE38. The number of huCD3<sup>+</sup> cells is determined  
6 by multiplying the total number of cells recovered  
7 from the LN's by the fraction of total cells  
8 (shown in Fig. 14A) that are huCD3<sup>+</sup>. (p<0.05 vs.  
9 untreated using a one-way ANOVA of ranks).

10 FIG. 15

11 Nucleotide and amino acid sequence of scFv(UCHT-  
12 1)-PE38. DNA sequence encoding the NcoI,  
13 HindIII, EcoRI, and BamHI/BglII restriction sites  
14 used for subcloning, are underlined; the flexible  
15 linker separating the V<sub>L</sub> from the V<sub>H</sub> domains is  
16 also underlined. Numbers correspond to  
17 nucleotides. Single letter codes denote encoded  
18 amino acids. The amino-terminal residues Met and  
19 Ala are encoded by the NcoI restriction site that  
20 was added to facilitate expression from the *E.*  
21 *coli* plasmid pET 15b. The 3' non-coding DNA  
22 between the EcoRI site and the BglII/BamHI site is  
23 carry-over sequence from the polylinker of an  
24 intermediate cloning vector (pLitmus 38, New  
25 England Biolabs).

26 FIG.16A-F

27 Schematic depiction of certain immunotoxin  
28 constructs according to the invention.  
29

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFICATION NOS.:

- SEQ. ID. NO:1 Amino acid sequence of scFv(UCHT-1)-PE38.  
V<sub>L</sub> = residues 3-111, linker = residues 112-127,  
V<sub>H</sub> = residues 128-249, connector plus truncated  
PE = residues 250-601.
- SEQ. ID. NO:2 Nucleotide sequence of scFv(UCHT-1)-PE38.
- SEQ. ID. NO:3 Amino acid sequence of native *Pseudomonas aeruginosa* exotoxin A (mature protein).
- SEQ. ID. NO:4 Signal sequence of *Pseudomonas aeruginosa* exotoxin A.
- SEQ. ID. NO:5 Linker (Gly<sub>3</sub>Ser)<sub>4</sub> of scFv(UCHT-1)-PE38.
- SEQ. ID. NO:6 Carboxy terminus of PE (ArgGluAspLeuLys).
- SEQ. ID. NO:7 Peptide sequence for PE (ArgGluAspLeu).
- SEQ. ID. NO:8 Peptide sequence for PE (LysAspGluLeu).
- SEQ. ID. NO:9 Connector peptide of scFv(UCHT-1)-PE38 (LysAlaSerGlyGly).
- SEQ. ID. NO:10 Diabody linker (Gly<sub>4</sub>Ser)
- SEQ. ID. NOS:  
11-22 Primers and oligos used in Example 1.

All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

1 Detailed Description of the Invention.

2  
3 1. CD3-Binding Domain.

4  
5 The term "CD3-binding domain" refers to an amino acid  
6 sequence capable of binding or otherwise associating with  
7 mammalian, and more preferably primate, and even more  
8 preferably, human, CD3 antigen on T cells or lymphocytes.  
9

10 The CD3-binding domain of the immunotoxins of the invention  
11 is preferably a polyclonal or monoclonal antibody to CD3, and  
12 more preferably, is a monoclonal anti-CD3 antibody. Even more  
13 preferably, the anti-CD3 antibody is a monoclonal antibody which  
14 is capable of binding an epitope on the  $\epsilon$  chain of human CD3, or  
15 alternatively an epitope formed by the  $\epsilon$  and  $\gamma$  chains of human  
16 CD3.  
17

18 The term "antibody" as used herein includes intact immuno-  
19 globulins as well as various forms of modified or altered  
20 antibodies, including fragments of antibodies, such as an Fv  
21 fragment, an Fv fragment linked by a disulfide bond, or a Fab or  
22 (Fab)'<sub>2</sub> fragment, a single chain antibody, and other fragments  
23 which retain the antigen binding function and specificity of the  
24 parent antibody. The antibody may be of animal (especially,  
25 mouse or rat) or human origin or may be chimeric or humanized.  
26 Methods of producing antibodies capable of binding specifically  
27 to CD3 antigen, and more particularly, human CD3 antigen, may be  
28 produced by hybridomas prepared using well-known procedures  
29 deriving from the work of Kohler and Milstein, *Nature*, 256:495-  
30 97 (1975).  
31

1 As is well-known in the art, an antibody "heavy" or "light"  
2 chain has an N-terminal variable region (V), and a C-terminal  
3 constant region (C). The variable region is the part of the  
4 molecule that binds to the antibody's cognate antigen, while the  
5 constant region determines the antibody's effector function.

6  
7 Full length immunoglobulin or antibody heavy chains  
8 comprise a variable region of about 116 amino acids and a  
9 constant region of about 350 amino acids. Full-length  
10 immunoglobulin or antibody light chains comprise an N-terminal  
11 variable region of about 110 amino acids, and a constant region  
12 of about 110 amino acids at the COOH-terminus.

13  
14 The heavy chain variable region is referred to as  $V_H$ , and  
15 the light chain variable region is referred to as  $V_L$ . Typically,  
16 the  $V_L$  will include the portion of the light chain encoded by the  
17  $V_L$  and  $J_L$  (i.e. joining region) gene segments (Sakans et al.  
18 (1979) *Nature* 280:288-294), and the " $V_H$ " will include the portion  
19 of the heavy chain encoded by the  $V_H$ ,  $D_H$  (i.e. diversity region)  
20 and  $J_H$  gene segments (Early et al. (1980) *Cell* 19:981-92).

21  
22 The term " $F(ab')_2$ " used hereinabove refers to a divalent  
23 fragment of an antibody including the hinge regions and the  
24 variable and first constant regions of the heavy and light  
25 chains, which can be produced by pepsin digestion of the native  
26 antibody molecule, or by recombinant means. The term "Fab"  
27 refers to a monovalent fragment of an antibody including the  
28 variable and first constant regions of the heavy and light  
29 chains, which can be generated by reducing the disulfide bridges  
30 of the  $F(ab')_2$  fragment, or by recombinant means.

31

1 The V<sub>H</sub> and V<sub>L</sub> fragments together are referred to as "Fv".  
2 The Fv region of an intact antibody is a heterodimer of (i.e.  
3 comprises on separate chains) the V<sub>H</sub> and the V<sub>L</sub> domains.  
4

5 As is well-known in the art, an immunoglobulin light or  
6 heavy chain variable region comprises three hypervariable  
7 regions, also called complementarity determining regions  
8 (CDR's), flanked by four relatively conserved "framework  
9 regions" (FR's).  
10

11 The combined framework regions of the constituent light and  
12 heavy chains serve to position and align the CDR's. The CDR's  
13 are primarily responsible for binding to an epitope of an  
14 antigen and are typically referred to as CDR1, CDR2 and CDR3,  
15 numbered sequentially starting from the N-terminus of the  
16 variable region chain. Framework regions are similarly  
17 numbered.  
18

19 Numerous framework regions and CDR's have been described  
20 (see, "Sequences of Proteins of Immunological Interest," E.  
21 Kabat and Wu, U.S. Government Printing Office, NIH Publication  
22 No. 91-3242 (1991) ("Kabat and Wu"). The CDR and FR polypeptide  
23 segments are designated empirically based on sequence analysis  
24 of the Fv region of preexisting antibodies or of the DNA  
25 encoding them. From alignment of antibody sequences of interest  
26 with those published in Kabat and Wu and elsewhere, framework  
27 regions and CDRs can be determined for the antibody or other CD3  
28 binding region of interest.  
29

30 By "chimeric" is generally meant a genetically engineered  
31 antibody comprising sequences derived from more than one natural  
32 antibody. An example of a chimeric antibody is one in which the



1 framework and complementarity determining regions are from  
2 different sources, as when a non-human variable domain is linked  
3 to a human constant domain. As a subset thereof, a "humanized"  
4 antibody is generally understood to comprise an antibody wherein  
5 non-human CDRs are integrated into framework regions at least a  
6 portion of which are human.

7  
8 As used herein, the term "single chain antibody" (or the  
9 term "single chain immunotoxin") refers to a molecule wherein  
10 the CD3-binding domain is on a single polypeptide chain.

11  
12 Single chain antibodies are typically prepared by  
13 determining and isolating the binding domain of each of the  
14 heavy and light chains of a binding antibody, and supplying a  
15 linking moiety which permits preservation of the binding  
16 function. This forms, in essence, a radically abbreviated  
17 antibody, having, on a single polypeptide chain, only that part  
18 of the variable domain necessary for binding to the antigen.  
19 Methods for preparation of single chain antibodies are described  
20 by Ladner et al., U.S. Patent No. 4,946,778, incorporated by  
21 reference.

22  
23 A single chain immunotoxin according to the invention  
24 comprises such a single chain antibody fragment. The toxin  
25 component is preferably fused to the CD3-binding domain(s),  
26 optionally via a linker peptide, but may also exist as a  
27 separate polypeptide chain linked via one or more disulfide  
28 bonds to the chain containing the CD3-binding domain.

29

1       An immunotoxin of the invention may be "monovalent," by  
2       which is meant that it contains one CD3-binding domain (e.g.,  
3       the combined V<sub>H</sub> and V<sub>L</sub> variable regions of an antibody) on the  
4       chain.

5  
6       An immunotoxin of the invention may also be "divalent," by  
7       which is meant that it contains two CD3-binding domains. The  
8       two antigen-binding domains can be located on a single chain, or  
9       alternatively, on two or more chains linked by disulfide bonds  
10      or otherwise in close association due to attractive forces  
11      (e.g., hydrogen bonds). When two CD3-binding domains are on a  
12      single chain, they may be present in tandem (i.e. following  
13      consecutively in series in the chain, bound together by a  
14      peptide bond or linker), or else separated on the chain by an  
15      intervening PE mutant, or other functional domains.

16  
17      Single chain antibodies (or single chain immunotoxins) may  
18      multimerize upon expression, depending on the expression system,  
19      by formation of interchain disulfide bonds with other single (or  
20      double) chain molecules, or by means of the intrinsic affinity  
21      of domains for their partner. The chains can form homodimers or  
22      heterodimers.

23  
24      The CD3-binding moiety of the immunotoxins of the invention  
25      is preferably a "recombinant" antibody. Likewise, the  
26      immunotoxins of the invention are "recombinant" immunotoxins.  
27      By the use of the term "recombinant" it is understood that the  
28      antibody (or immunotoxin) is synthesized in a cell from  
29      nucleotide (e.g., DNA) segments produced by genetic engineering.  
30      The term "isolated" indicates that a polypeptide has been  
31      removed from its native environment. A polypeptide produced

1 and/or contained within a recombinant host cell is considered  
2 isolated for purposes of the present invention. Also intended as  
3 an "isolated polypeptide" are polypeptides that have been  
4 purified, partially or substantially, from a recombinant host  
5 cell.

6

7 Preferably, the CD3-binding moiety of the immunotoxins of  
8 the invention is a single chain ("sc") antibody. The  
9 immunotoxin is preferably monovalent.

10

11 Most preferably, the CD3-binding moiety of the invention  
12 comprises a single chain Fv region (or CD3-binding fragment  
13 thereof) of an antibody, i.e. wherein the V<sub>H</sub> region (or CD3-  
14 binding portion thereof) is fused to the V<sub>L</sub> region (or CD3-  
15 binding portion thereof), optionally via a linker peptide.

16

17 The V<sub>L</sub> region is preferably linked via its carboxy terminus  
18 to the amino terminus of the V<sub>H</sub> region; alternatively, the V<sub>H</sub>  
19 region may be linked via its carboxy terminus to the amino  
20 terminus of the V<sub>L</sub> region.

21

22 Any peptide linker of the V<sub>L</sub> and V<sub>H</sub> regions preferably  
23 allows independent folding and activity of the CD3-binding  
24 domain; is free of a propensity for developing an ordered  
25 secondary structure which could interfere with the CD3-binding  
26 domain or cause immunologic-reaction in the patient, and has  
27 minimal hydrophobic or charged characteristic which could  
28 interact with the CD3-binding domain.

29

1 The peptide connector is preferably 1-500 amino acids; more  
2 preferably 1-250; and even more preferably no more than 1-100  
3 (e.g., about 1-25 or 10-20) amino acids.  
4

5 For each of the above preferences, the linker is preferably  
6 linear.  
7

8 In general, linkers comprising Gly, Ala and Ser can be  
9 expected to satisfy the criteria for such a peptide.  
10

11 For example, the linker in scFv(UCHT-1)-PE38, linking the  
12 carboxy terminus of the V<sub>L</sub> domain to the amino terminus of the V<sub>H</sub>  
13 domain, is [(Gly<sub>3</sub>)Ser]<sub>4</sub> (SEQ. ID. NO: 5).  
14

15 Examples of specific anti-CD3 antibodies the whole or  
16 fragments of which are suitable to be employed as a CD3-binding  
17 domain of the invention are:  
18

19 (1) UCHT-1 (Beverley P. C. L. and Callard, R. E. (1981)  
20 Eur. J. Immunol. 11: 329; and Burns, G. F. et al. (1982) J.  
21 Immunol. 129: 1451), the scFv sequence of which is included in  
22 SEQUENCE ID NO:1. UCHT-1 is a monoclonal mouse anti-human anti-  
23 CD3 antibody having an IgG1, Kappa isotype. The antibody reacts  
24 with T cells in thymus, bone marrow, peripheral lymphoid tissue,  
25 and blood. The intact antibody is commercially available from  
26 Biomeda (Catalog No. K009, V1035) or Coulter Corp. The  
27 variable regions comprise residues 3 to 112 (light chain) and  
28 128 to 249 (heavy chain) of SEQ. ID NO:1 herein. UCHT-1 is non-  
29 activating as an Fv fragment and has been used as a fusion  
30 partner with anti-HER2 bispecific immunoconjugates in targeting  
31 T-cells to human breast and ovarian tumor cells (see Shalaby et  
32 al. (1992), J. Exp. Med. 175:217).

1           (2) SP34 (first isolated by C. Terhorst, Beth Israel  
2 Deaconess Hospital), reacts with both primate and human CD3.  
3 SP34 differs from UCHT-1 and BC-3 (described below) in that SP-  
4 34 recognizes an epitope present on solely the  $\epsilon$  chain of CD3  
5 (see Salmeron et al., (1991) *J. Immunol.* 147: 3047) whereas  
6 UCHT-1 and BC-3 recognize an epitope contributed by both the  $\epsilon$   
7 and  $\gamma$  chains. The intact antibody is commercially available from  
8 PharMingen.

9  
10           (3) BC-3 (Fred Hutchinson Cancer Research Institute) (used  
11 in Phase I/II trials of GvHD) (Anasetti, et al., (1992)  
12 Transplantation 54: 844).

13  
14           Other monoclonal antibodies having specific binding  
15 affinity for CD3 antigen and having at least some sequences of  
16 human origin are considered to be within the scope of homologs  
17 of the abovementioned antibodies. These antibodies include:  
18 (1) a monoclonal antibody having complementarity-determining  
19 regions identical with, for example, UCHT-1 (or SP34 or BC3) and  
20 having at least one sequence segment of at least five amino  
21 acids of human origin; and (2) a monoclonal antibody competing  
22 with, e.g., UCHT-1, for binding to human CD3 antigen at least  
23 about 80%, and more preferably at least about 90%, as  
24 effectively on a molar basis as UCHT-1, and having at least one  
25 sequence segment of at least five amino acids of human origin.  
26 By "specific binding affinity" is meant binding affinity  
27 determined by noncovalent interactions such as hydrophobic  
28 bonds, salt linkages, and hydrogen bonds on the surface of  
29 binding molecules. Unless stated otherwise, "specific binding  
30 affinity" implies an association constant of at least about  $10^6$   
31 liters/mole for a bimolecular reaction.

1       Antibodies of this invention having complementarity-  
2       determining regions substantially homologous with those of,  
3       e.g., UCHT-1, are also within the scope of this invention and  
4       can be generated by in vitro mutagenesis. Among the mutations  
5       that can be introduced into constant or variable regions that  
6       substantially preserve affinity and specificity of such homologs  
7       are mutations resulting in conservative amino acid  
8       substitutions, such as are well-known in the art. With respect  
9       to UCHT-1, such mutant forms of antibodies preferably have  
10      variable regions which are at least 80% identical, and more  
11      preferably at least 90% identical, to the variable region of  
12      UCHT-1. Even more preferably, each of the complementarity-  
13      determining regions of such mutant forms of antibodies is at  
14      least 80%, and more preferably at least 90%, or at least 95%,  
15      identical to the corresponding complementarity-determining  
16      region of UCHT-1.

17  
18      As a practical matter, whether any particular polypeptide  
19      sequence is at least 80%, 90%, or at least 95%, "identical to"  
20      another polypeptide can be determined conventionally using known  
21      computer programs such the Bestfit program (Wisconsin Sequence  
22      Analysis Package, Version 8 for Unix, Genetics Computer Group,  
23      University Research Park, 575 Science Drive, Madison, Wis.  
24      53711). When using Bestfit or any other sequence alignment  
25      program to determine whether a particular sequence is, for  
26      instance, 95% identical to a reference sequence according to the  
27      present invention, the parameters are set, of course, such that  
28      the percentage of identity is calculated over the full length of  
29      the reference amino acid sequence and that gaps in homology of up  
30      to 5% of the total number of amino acid residues in the reference  
31      sequence are allowed.

32

1       The CD3 binding moiety of the invention in a preferred  
2       embodiment recognizes an epitope of human CD3 formed by both the  
3        $\gamma$  and  $\epsilon$  chains, and is preferably UCHT-1, and more preferably,  
4       is the Fv region (or CD3-binding fragment thereof) of UCHT-1.  
5

6       Even more preferably, the CD3 binding moiety is a single  
7       chain fragment of UCHT-1, and most preferably, is a single chain  
8       Fv region (or CD3-binding fragment thereof) of UCHT-1.  
9

10       It has been found that the Fv region of UCHT-1, when  
11       reconstituted as a single chain and fused to a cell-binding  
12       domain-deleted fragment of *Pseudomonas aeruginosa* exotoxin A,  
13       demonstrates high levels of potency in T-cell killing in  
14       standard in vitro assays and in vivo in transgenic mice  
15       heterozygous for human CD3 $\epsilon$ .  
16

17       2.    *Pseudomonas* toxin component.  
18

19       *Pseudomonas* exotoxin-A (hereinafter, "PE") is an extremely  
20       active monomeric protein of 613 amino acids (molecular weight  
21       66Kd), secreted by *Pseudomonas aeruginosa*, which inhibits  
22       protein synthesis in eukaryotic cells through inactivation of  
23       elongation factor 2 (EF-2), an essential eukaryotic translation  
24       factor by catalyzing its ADP-ribosylation (i.e. catalyzing the  
25       transfer of the ADP ribosyl moiety of oxidized NAD onto EF-2),  
26       see Kreitman and Pastan (1994) Blood 83: 426.  
27

28       The mature polypeptide has the amino acid sequence set  
29       forth in SEQ. ID NO:3 herein, which normally is preceded by a  
30       signal sequence of 25 residues as set forth in SEQ. ID NO:4.  
31

1 Three structurally distinct domains in native PE act in  
2 concert to promote cytotoxicity (see Pastan et al., U.S.  
3 4,892,827, incorporated by reference; see also U.S. 5,696,237  
4 and U.S. 5,863,745, also incorporated by reference). Domain  
5 Ia, at the amino terminus (and generally assigned residues 1 to  
6 about 252 of SEQ. ID NO:3), mediates cell targeting and binding.  
7 Domain II (at residues 253-364 of SEQ. ID NO:3) is responsible  
8 for translocation across the cell membrane into the cytosol; and  
9 Domain III (residues 405 to 613 of SEQ. ID NO:3) mediates ADP  
10 ribosylation of elongation factor 2, thereby inactivating the  
11 protein and causing cell death. Domain III contains a carboxy-  
12 terminal sequence (REDLK) (SEQ. ID. NO:6) that directs the  
13 endocytosed and processed toxin into the endoplasmic reticulum.  
14 While Domain Ib (residues 365-404 of SEQ. ID NO:3) appears to  
15 act in concert with Domain III, deletion of residues 365-380 of  
16 this domain results in no loss of activity.

17  
18 The "PE mutant" or, alternatively "PE component," of the  
19 immunotoxins of the invention is a mutant form of native PE  
20 having translocation and catalytic (i.e. ADP-ribosylating)  
21 functions but having substantially diminished or deleted cell-  
22 binding capability.

23  
24 Disruption or deletion of all or substantially all of cell-  
25 binding Domain Ia has been found to substantially reduce the  
26 cell-binding capability and thus the non-specific toxicity of  
27 the native PE molecule.

28  
29 For example, deletion of Domain Ia yields a 40 kDa protein,  
30 PE40, which itself is not cytotoxic despite retaining the  
31 translocation and ADP-ribosylation functions of domains II and  
32 III, respectively (Kondo et al., 1988, *J. Biol. Chem.*, 263:9470-  
33 9475).



1 PE38 is a 38 kDa fragment of PE also essentially lacking  
2 Domain Ia of the mature PE protein (e.g., lacking amino acids 1-  
3 250 of SEQ. ID. NO: 3), and also lacking amino acid residues 365  
4 to 380 of SEQ. ID. NO:3, and thus having the amino acid sequence  
5 comprising residues 251 to 364 joined to 381 to 613 of SEQ. ID  
6 NO:3 (see residues 255-601 of SEQ. ID. NO:1). See also U.S.  
7 Patent No. 5,608,039, col. 10, ll. 1-20, where PE38 is indicated  
8 to refer to a truncated toxin composed of amino acids 253-364 and  
9 381-613 of native PE. Advantageously, PE38 lacks the cysteine  
10 residues at positions 372 and 379 of the native protein, which  
11 otherwise can potentially form disulfide bonds with other  
12 cysteines during the renaturation process and can lead to  
13 formation of inactive chimeric toxins.  
14

15 A PE toxin component of the polypeptides of the invention  
16 may also comprise a polypeptide which is at least 90% identical  
17 to, and more preferably at least 95% identical to, and even more  
18 preferably at least 99% identical to, the sequence defined by  
19 residues 255-601 of SEQ. ID. NO:1, wherein the term "identical  
20 to" has the significance indicated previously.  
21

22 PE38KDEL has the amino acid sequence of PE38, described  
23 above, with the exception that the carboxyl terminus of the  
24 toxin is changed from the original sequence REDLK (SEQ. ID. NO:  
25 6) to KDEL (SEQ. ID. NO: 8).  
26

27 Other deletions or changes may be made in PE or in addition  
28 of a linker such as an IgG constant region connecting an  
29 antibody to PE, in order to increase cytotoxicity of the fusion  
30 protein toward target cells, or to decrease nonspecific  
31 cytotoxicity toward cells lacking the corresponding CD3 antigen.  
32 Deleting a portion of the amino terminal end of PE domain II  
33 increases cytotoxic activity, in comparison to the use of native

1 PE molecules or those where no significant deletion of domain II  
2 has occurred. Other modifications include an appropriate  
3 carboxyl terminal sequence to the recombinant PE molecule to  
4 help translocate the molecule into the cytosol of target cells.  
5 Amino acid sequences which have been found to be effective  
6 include REDLK (SEQ. ID. NO: 6) (as in native PE), REDL (SEQ. ID.  
7 NO:7) or KDEL (SEQ. ID. NO:8) (as in PE38KDEL discussed above),  
8 repeats of those, or other sequences that function to maintain  
9 or recycle proteins into the endoplasmic reticulum, see Pastan,  
10 U.S. Patent No. 5,489,525, incorporated by reference. Other  
11 mutants may comprise single amino acid substitutions (e.g.,  
12 replacing Lys with Gln at positions 590 and 606).  
13

14 Additional PE mutants having recognition moieties inserted  
15 into Domain III of PE are described by Pastan et al., U.S.  
16 Patent No. 5,458,878, incorporated by reference.  
17

### 18 3. Construction of Immunotoxins.

19  
20 This invention includes fusions of a CD3-binding domain to  
21 one or more *Pseudomonas* mutants; and also includes immunotoxin  
22 fusions comprising two or more CD3-binding domains and at least  
23 one PE mutant.  
24

25 The term "fused" or "fusion" as employed herein refers to  
26 polypeptides in which:  
27

- 28 (i) a "first polypeptide domain" is bound at its carboxy  
29 terminus via a chemical (i.e. peptide) bond to the amino  
30 terminus of a "second polypeptide domain," optionally via a  
31 peptide connector, or, conversely, where  
32 (ii) the "second polypeptide domain" of (i) is bound at its  
33 carboxy terminus via a chemical (i.e. peptide) bond to the amino

1 terminus of the "first polypeptide domain" of (i), optionally  
2 via a peptide connector.

3  
4 Similarly, "fused" when used in connection with the  
5 polynucleotide intermediates of the invention means that the 3'-  
6 [or, conversely, 5'-] terminus of a nucleotide sequence  
7 encoding a first functional domain is bound to the respective  
8 5'-[or conversely, 3'-] terminus of a nucleotide sequence  
9 encoding a second functional domain, either directly via a  
10 chemical (i.e. covalent) bond or indirectly via a connector  
11 nucleotide sequence which itself is chemically (i.e. covalently)  
12 bound to the first functional domain-encoding nucleotide  
13 sequence and the second functional domain-encoding nucleotide  
14 sequence via their termini.

15  
16 Additional peptide sequences making up the fusions may be  
17 selected from full length or truncated (e.g., soluble,  
18 extracellular fragments of) human proteins. Examples of such  
19 peptide sequences include human immunoglobulin protein domains,  
20 domains from other human serum proteins, or other domains which  
21 can be multimerized (see Kostelny et al., 1992, *J. Immunol.* 148:  
22 1547-1553; Tso et al., WO 93/11162; Pack and Pluckthun, 1992,  
23 *Biochemistry* 31: 1579-1584; Hu et al., 1996, *Can. Res.* 56: 3055-  
24 3061; Wu, WO 94/09817); Pack et al., 1995, *J. Mol. Biol.* 246: 28-  
25 34.

26  
27 Said additional functional domains may also serve as  
28 peptide connectors, for example, joining the CD3 antigen-binding  
29 domain to the PE component; or alternatively, said additional  
30 domain(s) may be located elsewhere in the fusion molecule, e.g.,  
31 at the amino or carboxy terminus thereof.

32

1 In a preferred embodiment of the invention, a single chain  
2 Fv of an anti-CD3 antibody is fused to a truncated fragment of  
3 PE having translocation and catalytic functions but  
4 substantially lacking cell binding capability.  
5

6 Preferably, the antibody binding regions which recognize  
7 the CD3 antigen may be inserted in replacement for deleted  
8 domain Ia of the PE molecule. Thus in the various embodiments of  
9 the invention, it is preferred that the CD3-binding moiety be  
10 linked via its carboxy terminus (optionally through a connector  
11 peptide or other functional domain) to the amino terminus of the  
12 PE toxin component.

13  
14 Alternatively, the PE toxin component may be linked via its  
15 carboxy terminus to the amino terminus of the CD3-binding moiety  
16 (also, optionally, via a connector peptide or other functional  
17 domain).

18  
19 Where there are multiple CD3-binding domains on a single  
20 chain, these may be linked in tandem by a peptide bond or  
21 linker, or else separated by an intervening PE component or  
22 another functional moiety.  
23

24 Any peptide connector linking the CD3-binding region and  
25 the PE component preferably allows independent folding and  
26 activity of the CD3-binding domain; is free of a propensity for  
27 developing an ordered secondary structure which could interfere  
28 with the CD3-binding domain or cause immunologic-reaction in the  
29 patient, and has minimal hydrophobic or charged characteristic  
30 which could interact with the CD3-binding domain.  
31

1       The connector is preferably 1-500 amino acids; more  
2 preferably 1-250; and even more preferably no more than 1-100  
3 (e.g., 1-25, 1-10, 1-7 or 1-4) amino acids.  
4

5       For each of the above preferences, the connector is  
6 preferably linear.  
7

8       In general, connector peptides linking the CD3-binding  
9 domain and the PE component which comprise small, uncharged  
10 amino acids can be expected to satisfy the criteria for such a  
11 connector. For example, the connector peptide in sc(UCHT-1)-  
12 PE38 is Lys-Ala-Ser-Gly-Gly (KASGG) (SEQ. ID. NO:9). Other  
13 peptides of various lengths and sequence composition may also be  
14 useful.  
15

16       Most preferably, the immunotoxin of the invention is a  
17 single chain polypeptide comprising the Fv region (or CD3-  
18 binding fragment thereof) of UCHT-1 fused via its carboxy  
19 terminus, optionally via a connector peptide, to the amino  
20 terminus of PE38.  
21

22       A schematic drawing of such a molecule is shown in Figure  
23 1. scFv(UCHT-1)-PE38 is a protein of 600 amino acids, having a  
24 predicted molecular weight of 64,563 daltons (64.5 kD).  
25

26       It will be noted that the actual translation product from  
27 E. coli of the molecule schematically depicted in Figure 1 may  
28 comprise an added N-terminal methionine (Met) residue, because  
29 of incomplete cleavage of the Met normally supplied to a coding  
30 sequence to initiate transcription from E. coli. Additionally,  
31 the scFv(UCHT-1)PE38 polypeptide prepared according to Example 1

1 may contain an added alanine (Ala) at the N-terminus or at  
2 position 2 (i.e. following Met) as a result of sequence added at  
3 the N-terminus to facilitate cloning. The mature amino terminus  
4 of the variable region of the light chain of UCHT-1 begins at  
5 position 3 of SEQ. ID. NO:1, i.e. aspartic acid (Asp).  
6 Accordingly, E. coli expression of the molecule depicted in  
7 Figure 1 as prepared according to Example 1 may yield one or  
8 more of the following functionally equivalent products,  
9 depending on the expression strain used, and the precise  
10 fermentation and purification conditions used: the polypeptide  
11 having sequence 1-601 of SEQ. ID. NO:1 and encoded by  
12 nucleotides 1-1803 of SEQ. ID. NO: 2; the polypeptide having  
13 sequence 2-601 of SEQ. ID. NO:1 and encoded by nucleotides 4-  
14 1803 of SEQ. ID. NO:2; and the polypeptide having sequence 3-601  
15 of SEQ. ID. NO:1 and encoded by nucleotides 7-1803 of SEQ. ID.  
16 NO:2.

17  
18 It shall be understood that any of such forms of the  
19 protein (or the corresponding nucleic acid) are encompassed by  
20 the term "scFv(UCHT-1)-PE38" as employed herein, unless  
21 otherwise indicated.

22  
23 This invention also encompasses polypeptides which are at  
24 least 80% identical to, and more preferably at least 90%  
25 identical to, and even more preferably, at least 95% identical  
26 to, the polypeptide having SEQ. ID. NO:1, wherein the term  
27 "identical to" has the meaning previously indicated.

28  
29 Certain immunotoxin molecules may be "dimerized" by the  
30 attractive forces between domains located on the polypeptide

1 chains or by the formation of disulfide bonds between cysteine  
2 residues.

3

4 For example, a dimer may be formed from two polypeptide  
5 chains, or from two pairs of chains. Dimers may be homodimers  
6 or heterodimers (An example of a heterodimer is a construct in  
7 which the PE toxin is present on only one of two chains.)

8

9 Certain divalent single chain immunotoxin constructs, or  
10 dimerized constructs, according to the invention are illustrated  
11 in Figure 16.

12

13 The dimerized immunotoxin constructs depicted in Figures  
14 16A, C, D, E and F comprise two (or more) chains. The  
15 construct depicted in Figure 16B is a divalent single chain  
16 immunotoxin. The molecules shown in Figure 16E are full length  
17 recombinantly prepared antibodies linked to a toxin. The  
18 construct of Figure 16F is a recombinantly prepared  $F(ab')_2$   
19 fragment (i.e. comprising a dimer of two pairs of chains) linked  
20 to toxin.

21

22 The PE toxin in the constructs depicted in Figure 16 is  
23 preferably PE38, and the antibody variable domains may be  
24 derived from UCHT-1.

25

26 In particular, a first illustrative embodiment of a dimeric  
27 immunotoxin of the invention is a diabody, as illustrated in  
28 Figure 16A.





1 i.e. by a peptide bond or via a peptide linker which is  
2 optionally flexible.

3  
4 Figure 16B depicts a construct having the configuration:  $V_L$   
5 - L -  $V_H$  - X -  $V_L$  - L -  $V_H$  - Y - Toxin, wherein X and Y are  
6 independently selected from a peptide bond or linker. In  
7 particular, L may be a linker such as that depicted in Figure 1  
8 hereof, i.e. (GGGS)<sub>4</sub> (SEQ. ID. NO:5), and each of X and Y may  
9 have a sequence such as that of the "connector" also described in  
10 Fig. 1 (i.e. KASGG, SEQ. ID. NO:9).

11  
12 Similar to the construct shown in Figure 1, the  $V_L$  and  $V_H$   
13 domains of each of the two Fv regions are separated by a peptide  
14 linker L which is flexible (represented in Figure 16B, as well as  
15 in Figures 16C and D, by a looping line connecting each  $V_L$  and  $V_H$   
16 domain), having preferably about 10-30, and more preferably about  
17 14 to 25, amino acids.

18  
19 Preferably, the two Fv regions in the construct shown in  
20 Figure 16B are both anti-CD3 binding domains. Thus in one  
21 embodiment, the Fv regions may bind to the same epitope of CD3,  
22 and may even be identical (or each region or its encoding  
23 nucleotide sequence may be modified to facilitate expression or  
24 inhibit recombination); or alternatively, each Fv may be selected  
25 to bind to a different epitope on human CD3 antigen.

26  
27 A PE toxin component of the invention may be linked  
28 (optionally through intervening linkers or functional sequences)  
29 to the carboxy or the amino terminus of one of the Fv domains.  
30 (Alternatively, multiple PE toxin segments may be present in the  
31 molecule.) In Figure 16B, the PE sequence is linked to the  
32 carboxy terminus of one of the Fv domains.

33

1 Tandem single chain antibody molecules in which the antigen  
2 binding regions bind to different antigens, rendering such  
3 molecules "bispecific", are described in general by Gruber et al.  
4 (1994) *J. Immunol.* 152: 5368, Kurcucz and Segal (1995) *J.*  
5 *Immunol.* 154: 4576, Mallender et al., (1994) *J. Biol. Chem.* 269:  
6 199, and Mack et al. (1995) *Proc. Nat. Acad. Sci.* 92: 7021.  
7

8 Still another construct of the invention is prepared from  
9 two polypeptide chains each comprising a "dimerizing domain"  
10 which serves to facilitate dimerization between the chains by  
11 associational forces (e.g., hydrogen bonding), rather than by  
12 disulfide bonding. (The mentioned associational forces are  
13 represented by the dots in Figure 16C, as well as in Figure 16D.)  
14 Each dimerizing domain, depicted in Figure 16C by a pair of  
15 stars, can be located internally within the chain, for example,  
16 between the Fv region and the PE toxin component (as shown); or  
17 in another aspect, the dimerizing domain may be located at the  
18 N-terminus of the Fv region (not shown); and in still another  
19 aspect, the dimerizing domain may be located at the C-terminus of  
20 the PE toxin (not shown). In the construct depicted in Figure  
21 16C, each chain has the configuration:  $V_L$ - L -  $V_H$ - dimerizing  
22 domain - PE mutant toxin.  
23

24 Dimerizing domains are described in general by Pack and  
25 Pluckthun (1992) *Biochem.* 31: 1579 and Kostelny et al., supra.  
26 Suitable dimerizing domains may be derived from heterodimeric  
27 transcription factors or amphiphilic helices, and expressed in  
28 mammalian cells as well as *E. coli*.  
29  
30

31 Another dimerized construct according to the invention is  
32 prepared from single chain immunotoxins comprising the hinge and  
33 third constant region ("CH3") of Ig to effect dimerization

1 through formation of disulfide bonds and attractive forces  
2 between the CH3 segments.

3  
4 As shown in Figure 16D, a "minibody"-toxin of the invention  
5 may comprise two (e.g., identical) single chains, each of which  
6 chains comprises an Fv region linked via hinge ("H") and CH3 of,  
7 e.g., human IgG1, to the PE toxin component. Each of the lightly  
8 shaded ovals in Figure 16D represents the hinge and CH3 domains.  
9 Thus each chain has the configuration:  $V_L - L - V_H - H + CH3 - PE$   
10 mutant toxin. The polypeptide chains are linked by disulfide  
11 bonds (represented in Figure 16D, as well as in Figures 16E and  
12 F, by thickened lines) as well as associational forces  
13 (represented by dots), between the respective hinge and CH3  
14 domains. (A variant construct referred to in Figure 16D as " $\Delta$   
15 minibody-toxin" is mutated to prevent mispairing of cysteines by  
16 replacing the cysteine in the hinge region which ordinarily pairs  
17 the heavy and light chains of the native antibody, with, e.g.,  
18 serine or alanine, and leaving intact the two remaining cysteines  
19 in the hinge which bind the heavy chains.)

20  
21 Other variants utilize the hinge from other immunoglobulin  
22 isotypes or other mammalian species, e.g., murine IgG's. A  
23 "minibody" has been described in general by Hu et al. (1996) *Can.*  
24 *Res.* 56: 3055.

25  
26 Another illustrative construct according to the invention  
27 comprises a recombinant antibody fused via the C-terminus of  
28 either the heavy chain (Fig. 16E, left panel) or the light chain  
29 (Fig. 16E, right panel) to a PE mutant toxin according to the  
30 invention. As in the native antibody, the chains are linked by  
31 disulfide bonds (thickened lines connecting chains), as shown.  
32 Said full length antibody toxins generally dimerize in pairs. In  
33 such constructs, a non-huFc $\gamma$ -receptor binding Ig, such as murine

1 IgG2b or human IgG<sub>4</sub>, may be substituted for the native Fc.  
2 Optionally, a PE toxin component may be present on both heavy and  
3 light chains (not shown).  
4

5 An additional construct according to the invention comprises  
6 a recombinantly prepared F(ab')<sub>2</sub> fragment (including the  
7 indicated hinge region), which is linked via the carboxy terminus  
8 of the heavy chain (Fig. 16F, left panel) or light chain (Fig.  
9 16F, right panel) (optionally via a linker, not shown), to a PE  
10 mutant toxin. Said F(ab')<sub>2</sub> toxin molecules generally dimerize in  
11 pairs. (The lightly shaded ovals in Figure 16F represent either  
12 the constant domain of the heavy chain ("C<sub>H</sub>") or the constant  
13 domain of the light chain ("C<sub>L</sub>"), as indicated. The hinge regions  
14 of the polypeptide chain are separately represented from the  
15 constant regions by the disulfide-linked connectors labelled  
16 "hinge". Thus, the respective chains have the configuration V<sub>L</sub>  
17 - C<sub>L</sub> and V<sub>H</sub> - C<sub>H1</sub> - hinge - PE toxin (Figure 16F, left) or,  
18 alternatively, V<sub>L</sub> - C<sub>L</sub> - PE toxin and V<sub>H</sub> - C<sub>H1</sub> - hinge (Figure  
19 16F, right).  
20

21 The above constructs can be prepared from known starting  
22 materials by techniques of recombinant engineering known by  
23 workers skilled in the art.  
24

25  
26 The invention is also intended to include polypeptide  
27 homologs (and the DNA molecules which encode said polypeptides)  
28 which differ from a disclosed species of polypeptide by having,  
29 for example, conservative substitutions in amino acid over the  
30 disclosed polypeptide, or minor deletions or additions of  
31 residues not otherwise substantially affecting the CD3-binding  
32 ability or catalytic activity of the immunotoxin.  
33

1 By "conservative substitution" is meant the substitution of  
2 one or more amino acids by others having similar properties such  
3 that one skilled in the art of polypeptide chemistry would  
4 expect at least the secondary structure, and preferably the  
5 tertiary structure of the polypeptide to be substantially  
6 unchanged. Conservative replacements are generally those that  
7 take place within a family of amino acids that are related in  
8 their side chains. Typical amino acid replacements include  
9 alanine or valine for glycine, asparagine for glutamine, serine  
10 for threonine and arginine for lysine.

11  
12 Also within the scope of this invention are homologs of the  
13 species of immunotoxin disclosed herein.

14  
15 The term "homolog" or "homology" refers to sequence  
16 similarity between two peptides or between two nucleic acid  
17 molecules. Homology can be determined by comparing a position in  
18 each sequence which may be aligned for purposes of comparison.  
19 When a position in the compared sequence is occupied by the  
20 identical base or amino acid, then the molecules are homologous  
21 at that position. A degree of homology between sequences is a  
22 function of the number of matching or homologous positions shared  
23 by the sequences.

24  
25 Preferably, any homolog of an immunotoxin polypeptide  
26 species of the invention is at least 80% identical to, and  
27 preferably at least 90% identical to, and more preferably at  
28 least 95% identical to, said immunotoxin polypeptide of the  
29 invention.

30  
31 All of the amino acids of the polypeptides of the invention  
32 (except for glycine) are preferably naturally-occurring L-amino  
33 acids.

1       Also within the scope of this invention are isolated  
2 polynucleotides (e.g., cDNA) encoding the recombinant  
3 immunotoxin polypeptides of the invention and their homologs,  
4 and in particular, polynucleotides encoding sc(UCHT-1)-PE38  
5 having residues 1-601, 2-601 or 3-601 of SEQ. ID NO:1, or  
6 fragments of sc(UCHT-1)-PE38 having at least 100 (and preferably  
7 at least 200) amino acids.  
8

9       This invention includes not only the nucleic acid depicted  
10 in SEQ. ID NO:2, but also isolated nucleic acids encoding the  
11 polypeptide of SEQ. ID. NO:1 or a fragment thereof and having a  
12 sequence which differs from the nucleotide sequence shown in  
13 SEQ. ID NO:2 due to the degeneracy of the genetic code; as well  
14 as complementary strands of the foregoing nucleic acids.  
15

16       Another aspect of the invention provides a polynucleotide  
17 (having preferably at least 300 bases (nucleotides), and more  
18 preferably at least 600 bases, and even more preferably at least  
19 900 bases) which hybridizes to a polynucleotide which encodes a  
20 polypeptide of the invention, such as the polypeptide of SEQ.  
21 ID. NO:1. Said hybridization reaction may be carried out under  
22 under low or high stringency conditions.  
23

24  
25       Appropriate stringency conditions which promote DNA  
26 hybridization (for example, 6.0 x sodium chloride/sodium nitrate  
27 (SSC) at about 45°C. followed by a wash of 2.0xSSC at 50°C.), are  
28 known to those skilled in the art or can be found in Current  
29 Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989),  
30 6.3.1-6.3.6. For example, the salt concentration in the wash  
31 step can be selected from a low stringency of about 2.0xSSC at 50  
32 °C. to a high stringency of about 0.2xSSC at 50°C. In addition,  
33 the temperature in the wash step can be increased from low

1 stringency conditions at room temperature, about 22°C. to high  
2 stringency conditions at about 65°C.  
3

4 By the term "stringent hybridization conditions" is intended  
5 overnight incubation at 42° C. in a solution comprising: 50%  
6 formamide, 5 x SSC 750 mM NaCl, 75 mM trisodium citrate, 50 mM  
7 sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran  
8 sulfate, and 20 mu g/ml denatured, sheared salmon sperm DNA,  
9 followed by washing the filters in 0.1 x SSC at about 65° C.  
10

11 By "isolated" polynucleotide(s) is intended a nucleic acid  
12 molecule, DNA or RNA, which has been removed from its native  
13 environment. For example, recombinant DNA molecules contained in  
14 a vector are considered isolated for the purposes of the present  
15 invention. Further examples of isolated DNA molecules include  
16 recombinant DNA molecules maintained in heterologous host cells  
17 or purified (partially or substantially) DNA molecules in  
18 solution. Isolated RNA molecules include in vivo or in vitro RNA  
19 transcripts of the DNA molecules of the present invention.  
20 Isolated nucleic acid molecules according to the present  
21 invention further include such molecules produced synthetically.  
22  
23

24 The invention also includes isolated oligonucleotides  
25 encoding the connector peptides and/or linker of the invention.  
26 Such oligonucleotides should be "fused in frame" with the  
27 polynucleotides encoding the CD3-binding domain and PE  
28 component, and preferably include restriction sites unique in  
29 the molecule.  
30

31 By "fused in frame" is meant that: (1) there is no shift  
32 in reading frame of the CD3-binding domain or the PE component  
33 caused by the linker oligonucleotide; and (2) there is no

1 translation termination between the reading frames of the CD3-  
2 binding domain and the PE component.

3  
4 This invention further encompasses physiologically  
5 functional equivalent proteins of the novel fusion polypeptides  
6 which are intermediates in the synthesis of the novel  
7 polypeptides.

8  
9 The term "physiologically functional equivalent" refers to  
10 a larger molecule comprising the fusion polypeptide of the  
11 invention to which has been added such amino acid sequence as is  
12 necessary or desirable for effective expression and secretion of  
13 the mature recombinant fusion polypeptide of the invention from  
14 a particular host cell.

15  
16 Such added sequence is typically at the amino terminus of  
17 the mature protein, and usually constitutes a leader (i.e.  
18 signal) sequence which serves to direct the proteins into the  
19 secretory pathway, and is normally cleaved from the protein at  
20 or prior to secretion of the protein from the cell.

21  
22 The signal sequence can be derived from the natural N-  
23 terminal region of the relevant protein, or it can be obtained  
24 from host genes coding for secreted proteins, or it can derive  
25 from any sequence known to increase the secretion of the  
26 polypeptide of interest, including synthetic sequences and all  
27 combinations between a "pre" and a "pro" region. The juncture  
28 between the signal sequence and the sequence encoding the mature  
29 protein should correspond to a site of cleavage in the host.

30  
31 In the polypeptides of the invention wherein a CD3-binding  
32 region leads expression, i.e. is upstream from other coding  
33 sequences in the fusion molecule, it may be expedient to utilize



1 a signal sequence to effectively obtain expression from  
2 mammalian systems (e.g., CHO, COS), or yeast (e.g., *P.*  
3 *pastoris*).  
4

5 However, the additional signal sequence is not necessarily  
6 that of the native immunoglobulin chain and may be obtained from  
7 any suitable source, provided it is suitable to effect  
8 expression/secretion of the mature polypeptide from the  
9 particular host cell.  
10

11 The addition of other sequences for facilitation of  
12 purification at the amino or carboxy terminus of the protein is  
13 contemplated as part of the invention. Examples of such  
14 sequences include poly-histidine tags for purification on nickel  
15 affinity resins and peptide sequences for recognition by  
16 antibodies against c-myc, or hemagglutinin (HA). Such peptide  
17 "tags" are familiar to those skilled in the art.  
18

19 In immunotoxin polypeptides of the invention wherein a PE  
20 toxin component leads expression, a suitable leader sequence may  
21 comprise the native PE exotoxin A leader sequence (SEQ. ID.  
22 NO:4) to accomplish secretion of the mature heterologous  
23 polypeptide from E.coli, mammalian (e.g., CHO, COS) cells or  
24 yeast. However, other leader sequence, not necessarily native  
25 to PE or to the host cell, may provide effective expression of  
26 the mature fusion protein in certain hosts.  
27

28 4. Methods for Preparation of Recombinant Immunotoxins of the  
29 Invention. - In General.

30 a. Preparation of antibody derived CD3-binding moiety.  
31

1 The general strategy for cloning one or more regions of an  
2 antibody begins by extracting RNA from the hybridoma cells, and  
3 reverse transcribing the RNA using random hexamers as primers.  
4

5 In particular, in order to clone the Fv fragment of an  
6 antibody, each of the  $V_H$  and  $V_L$  domains is amplified by polymerase  
7 chain reactions (PCR). Heavy chain sequences can be amplified  
8 using 5'-end primers designed according to the amino-terminal  
9 protein sequences of the heavy chain and 3' primers according to  
10 consensus immunoglobulin constant region sequences (Kabat and  
11 Wu, *supra*).  
12

13 Light chain Fv regions are amplified using 5'-end primers  
14 designed according to the amino-terminal protein sequences of  
15 the antibody light chain, and in combination with the primer C-  
16 kappa. Suitable primers for isolating the Fv region of UCHT-1  
17 are illustrated in Table I of Example 1, although one of skill  
18 in the art would recognize that other suitable primers may be  
19 derived from the sequence listings provided herein.  
20

21 The crude PCR products are subcloned into a suitable  
22 cloning vector. Clones containing the correct size insert by  
23 DNA restriction are identified. The nucleotide sequence of the  
24 heavy or light chain coding regions may then be determined from  
25 double stranded plasmid DNA using sequencing primers adjacent to  
26 the cloning site. Commercially available kits (e.g., the  
27 Sequenase kit, U.S. Biochemical Corp., Cleveland, Ohio, USA) may  
28 be used to facilitate sequencing the DNA.  
29

30 It will also be appreciated that, given the sequence  
31 information disclosed herein, one of ordinary skill in the art  
32 may readily prepare nucleic acids encoding these sequences using  
33 well-known methods. Thus, DNA encoding the Fv regions may be

1 prepared by any suitable method, including, for example,  
2 amplification techniques such as ligase chain reaction (LCR) and  
3 self-sustained sequence replication, cloning and restriction of  
4 appropriate sequences or direct chemical synthesis, such as by  
5 the phosphotriester method, the phosphodiester method, the  
6 diethylphosphoramidite method and the solid support method.  
7 Chemical synthesis produces a single stranded oligonucleotide.  
8 This may be converted into double stranded DNA by hybridization  
9 with a complementary sequence, or by polymerization with a DNA  
10 polymerase using the single strand as a template. While it is  
11 possible to chemically synthesize an entire single chain Fv  
12 region, it is preferable to synthesize a number of shorter  
13 sequences (about 100 to 150 bases) that are later ligated  
14 together.

15  
16 Alternatively, subsequences may be cloned and the  
17 appropriate subsequences cleaved using appropriate restriction  
18 enzymes. The fragments may then be ligated to produce the  
19 desired DNA sequence.

20  
21 Once the Fv variable light and heavy chain DNA is obtained,  
22 the sequences may be ligated together, either directly or  
23 through a DNA sequence encoding a peptide linker, or by PCR,  
24 using techniques well known to those of skill in the art. In a  
25 preferred embodiment, heavy and light chain regions are  
26 connected by a flexible peptide linker which starts at the  
27 carboxyl end of the light chain Fv domain and ends at the amino  
28 terminus of the heavy chain Fv domain. The entire sequence  
29 encodes the Fv domain in the form of a single-chain CD3-binding  
30 moiety.

1           b.    Fusion of CD3-binding region and PE Component.

2

3           The Fv region may be fused directly to the toxin moiety or  
4 may be joined through a connector peptide. The connector  
5 peptide may be employed simply to provide space between the  
6 antibody and the toxin moiety or to facilitate mobility between  
7 these regions to enable them to each attain their optimum  
8 conformation. The DNA sequence comprising the connector peptide  
9 may also provide sequences (such as primer sites or restriction  
10 sites) to facilitate cloning or may preserve the reading frame  
11 between the sequence encoding the antibody and the toxin moiety.

12

13           In general, the cloning of an immunotoxin fusion protein  
14 according to the invention involves separately preparing the DNA  
15 encoding the CD3-binding moiety and the DNA encoding the PE  
16 toxin  
17 moiety, and recombining the DNA sequences in a plasmid or other  
18 vector to form a construct encoding the particular desired  
19 fusion protein. The vector can be an expression plasmid  
20 containing appropriate promoter sequence, etc., or the  
21 immunotoxin-encoding DNA fragment can be subsequently  
22 transferred into an expression plasmid. Another approach  
23 involves inserting the DNA encoding the CD3-binding moiety into  
24 a construct already encoding the PE toxin moiety.

25

26       c.    Expression of recombinant immunotoxin.

27

28           Proteins of the invention can be expressed in a variety of  
29 host cells, including E. coli, other bacterial hosts, yeast, and  
30 various higher eucaryotic cells such as the COS, CHO and HeLa  
31 cell lines and myeloma cell lines. The recombinant protein gene  
32 will be operably linked to appropriate expression control  
33 sequences for each host. For E. coli, this includes a promoter

1 such as the T7, trp, tac, lac or lambda promoters, a ribosome  
2 binding site, and preferably a transcription termination signal.  
3 For eucaryotic cells, the control sequences will include a  
4 promoter and preferably an enhancer derived from immunoglobulin  
5 genes, SV40, cytomegalovirus, etc., and a polyadenylation  
6 sequence, and may include splice donor and acceptor sequences.  
7

8 Both diphtheria toxin and *Pseudomonas* exotoxin prevent  
9 protein synthesis in eucaryotic cells by ADP-ribosylation of  
10 elongation factor-2 (EF-2), an essential eucaryotic translation  
11 factor. Therefore, for eucaryotic expression, it is preferable  
12 that cells in which EF-2 is mutated and therefore resistant to  
13 ADP-ribosylation by P. exotoxin be utilized. Such mutant hosts  
14 and mutant EF-2 proteins have been described for both mammalian  
15 (Moehring et al., 1979 *Somatic Cell Genetics* 5: 469-480; Kohno et  
16 al., 1987, *J. Biol. Chem.* 262: 12298-12305) and yeast cells (Phan  
17 et al., 1993, *J. Biol. Chem.* 268:8665-8668; Kimata, et al., 1993,  
18 *Biochem. Biophys. Res. Commun.* 191: 1145-1151).  
19

20 The plasmids of the invention can be transferred into the  
21 chosen host cell by well-known methods such as calcium chloride  
22 transformation for E. coli and calcium phosphate treatment or  
23 electroporation for mammalian cells. Cells transformed by the  
24 plasmids can be selected by resistance to antibiotics conferred  
25 by genes contained on the plasmids, such as the amp, gpt, neo and  
26 hyg genes.  
27

28 It is apparent that modifications can be made to the single  
29 chain Fv region and fusion proteins comprising the single chain  
30 Fv region without diminishing their biological activity. Some  
31 modifications may be made to facilitate the cloning, expression,  
32 or incorporation of the single chain Fv region into a fusion  
33 protein. Such modifications are well known to those of skill in

1 the art and include, for example, a methionine added at the  
2 amino terminus to provide an initiation site, or additional  
3 amino acids placed on either terminus to create conveniently  
4 located restriction sites or termination codons. For example,  
5 the primers used in Example 1 introduce a sequence encoding an  
6 initiator methionine for expression in E. coli, and BamHI, XbaI,  
7 SalI, NcoI and BstXI restriction sites to facilitate cloning.  
8

9 Once expressed, the recombinant proteins can be purified  
10 according to standard procedures of the art, including ammonium  
11 sulfate precipitation, affinity columns, column chromatography,  
12 gel electrophoresis, and the like.  
13

14 Substantially pure compositions of at least about 90 to 95%  
15 homogeneity are preferred, and compositions having 98 to 99%, or  
16 greater than 99%, homogeneity are most preferred for  
17 pharmaceutical uses. Once purified, partially or to homogeneity  
18 as desired, the polypeptides should be substantially free of  
19 endotoxin for pharmaceutical purposes and may be used  
20 therapeutically.  
21

22 One of skill in the art would recognize that after chemical  
23 synthesis, biological expression, or purification, the single  
24 chain Fv region or a fusion protein comprising a single chain Fv  
25 region may possess a conformation substantially different from  
26 that of the native protein. In this case, it may be necessary  
27 to denature and reduce the protein and then to cause the protein  
28 to re-fold into the preferred conformation.  
29

30 Methods for expressing single chain antibodies and/or  
31 denaturing the protein and inducing refolding to an appropriate  
32 folded form, including single chain antibodies, from bacteria  
33 such as E. coli, have been described and are well-known and are

1 applicable to the polypeptides of this invention. See, Buchner  
2 et al., Analytical Biochemistry 205:263-270(1992).  
3

4 In particular, functional protein from E. coli or other  
5 bacteria is often generated from inclusion bodies and requires  
6 the solubilization of the protein using strong denaturants, and  
7 subsequent refolding. In the solubilization step, a reducing  
8 agent must be present to dissolve disulfide bonds as is well-  
9 known in the art. An exemplary buffer with a reducing agent is:  
10 0.1 M Tris, pH8, 6M guanidine, 2mM EDTA, 0.3M DTE  
11 (dithioerythritol). Reoxidation of protein disulfide bonds can  
12 be effectively catalyzed in the presence of low molecular weight  
13 thiol reagents in reduced and oxidized form, as described by  
14 Buchner et al.(1992), supra.  
15

16 Renaturation is typically accomplished by dilution (e.g.,  
17 100-fold) of the denatured and reduced protein into refolding  
18 buffer. Renaturation in the presence of 8mM GSSG has been found  
19 to provide a reproducible, highly stable product. An exemplary  
20 buffer for this purpose is 0.1 M Tris, pH 8.0, 0.5 M L-arginine,  
21 8mM oxidized glutathione (GSSG), and 2mM EDTA.  
22

23 5. Therapeutic Uses of Recombinant Anti-CD3 Immunotoxins.  
24

25 The immunotoxin polypeptides described herein are utilized  
26 to effect at least partial T-cell depletion in order to treat or  
27 prevent T-cell mediated diseases or conditions of the immune  
28 system. The immunotoxins may be utilized in methods carried out  
29 in vivo, in order to systemically reduce populations of T cells  
30 in a patient. The immunotoxins may also be utilized ex vivo in  
31 order to effect T-cell depletion from a treated cell population.

1           In vivo Applications

2  
3           It is within the scope of the present invention to provide  
4 a prophylaxis or treatment of T-cell mediated diseases or  
5 conditions by administering immunotoxin to a patient in vivo for  
6 the purpose of systemically killing T cells in the patient, and  
7 as a component of a preparation or conditioning regimen or  
8 induction tolerance treatment in connection with bone marrow or  
9 stem cell transplantation, or solid organ transplantation from  
10 either a human (allo-) or non-human (xeno-) source.

11  
12           Both B and T lymphocytes originate in the bone marrow from  
13 a common lymphoid progenitor, the pluripotent stem cell, but  
14 only B lymphocytes mature in the bone marrow. The T lymphocytes  
15 migrate to the thymus to undergo maturation, and then enter the  
16 bloodstream,  
17 from which they migrate to the peripheral lymphoid tissues. The  
18 lymphoid tissues include the central lymphoid organs where  
19 lymphocytes are generated, and secondary or peripheral lymphoid  
20 organs, where adaptive immune responses are initiated. The  
21 central lymphoid organs are the bone marrow and thymus. The  
22 peripheral lymphoid organs include the lymph nodes, the spleen,  
23 the gut-associated lymphoid tissues, the bronchial-associated  
24 lymphoid tissue and mucosal-associated lymphoid tissue. Janeway  
25 and Travers, *supra*, at §1-2.

26  
27           This invention comprises a method of treatment or  
28 prophylaxis of T-cell mediated disorders in a patient,  
29 comprising administering to a patient in need thereof a T-cell  
30 depleting effective amount of an immunotoxin of the invention.

31  
32           Depletion of the levels of T cells in the bone marrow, the  
33 peripheral blood and/or lymphoid tissues of the patient can



1 ameliorate the patient's T-cell mediated response to antigen,  
2 and assist in tolerance induction.

3  
4 For example, the immunotoxins can usefully be administered  
5 to a patient who is or will be a recipient of an allotransplant  
6 (or xenotransplant), in order to effect T-cell depletion in the  
7 patient and thereby prevent or reduce T-cell mediated acute or  
8 chronic transplant rejection of the transplanted allogeneic (or  
9 xenogeneic) cells, tissue or organ in the patient, or to permit  
10 the development of immunological tolerance to the cells, tissue  
11 or organ.

12  
13 Preferably, when administered in vivo to prevent or treat  
14 organ transplant rejection, it is desirable that the immunotoxin  
15 be administered to the patient over time in several doses. In  
16 general, it is preferred that at least the first dose precede  
17 the transplant  
18 surgery (preferably as long in advance as possible), and a  
19 subsequent dose or doses begin at the time of or shortly  
20 following the surgery.

21  
22 The immunotoxins can be administered in vivo either alone or  
23 in combination with other pharmaceutical agents effective in  
24 treating acute or chronic transplant rejection including  
25 cyclosporin A, cyclosporin G, rapamycin, 40-O-2-hydroxyethyl-  
26 substituted rapamycin (RAD), FK-506, mycophenolic acid,  
27 mycophenolate mofetil (MMF), cyclophosphamide, azathioprene,  
28 brequinar, leflunamide, mizoribine, deoxyspergualines, 2-amino-2-  
29 [2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride (FTY 720),  
30 corticosteroids (e.g., methotrexate, prednisolone,  
31 methylprednisolone, dexamethasone), or other immunomodulatory  
32 compounds (e.g., CTLA4-Ig); anti-LFA-1 or anti-ICAM antibodies,  
33 or other antibodies that prevent co-stimulation of T cells, for

1 example antibodies to leukocyte receptors or their ligands (e.g.,  
2 antibodies to MHC, CD2, CD3, CD4, CD7, CD25, CD28, B7, CD40,  
3 CD45, CD58, CD152 (CTLA-4), or CD 154 (CD40 ligand).

4  
5 In particular, prolonged graft acceptance and even apparent  
6 immunologic tolerance can be achieved by combined administration  
7 of an anti-CD3 immunotoxin of the invention and a spergualin  
8 derivative, such as a deoxyspergualine compound, or other  
9 spergualin analog, and this invention in a preferred embodiment  
10 comprises the combined administration of anti-CD3 immunotoxin and  
11 a deoxyspergualine compound in a tolerance induction regimen, see  
12 for example, Eckhoff et al., abstract presented to American  
13 Society of Transplant Surgeons, May 15, 1997, and Contreras, et  
14 al., (1998) Peritransplant tolerance induction with anti-CD3  
15 immunotoxin : A matter of proinflammatory cytokine control.  
16 Transplantation 65: 1159, both incorporated by reference. The  
17 term "deoxyspergualine compound" includes 15-deoxyspergualin  
18 (referred to as "DSG", and also known as gusperimus), i.e. i.e.  
19 N-[4-(3-aminopropyl) aminobutyl]-2-(7-N-guanidinoheptanamido)-2-  
20 hydroxyethanamide, and its pharmaceutically acceptable salts, as  
21 disclosed in U.S. Patent No. 4,518,532, incorporated by  
22 reference; and in particular (-)-15-deoxyspergualin and its  
23 pharmaceutically acceptable salts as disclosed in U.S. Patent No.  
24 4,525,299, incorporated by reference. The optically active (S)-  
25 (-) or (R)-(+)-15-deoxyspergualin isomers and salts thereof are  
26 disclosed in U.S. Patent No. 5,869,734 and EP 765,866, both  
27 incorporated by reference; and the trihydrochloride form of DSG  
28 is disclosed in U.S. Patent No. 5,162,581, incorporated by  
29 reference.

30  
31 Other spergualin derivatives for use with anti-CD3  
32 immunotoxin in a tolerance induction regimen include compounds

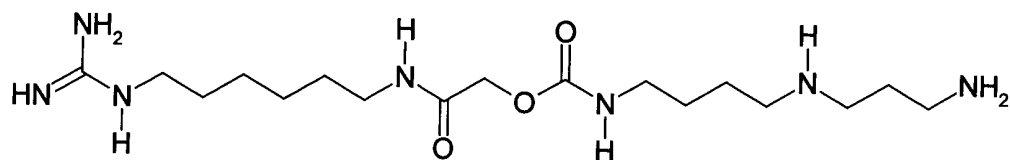
1 disclosed in U.S. Patent Nos. 4,658,058, 4,956,504, 4,983,328,  
2 4,529,549,; and EP 213,526, EP 212,606, all incorporated by  
3 reference.

4

5 The invention in a further preferred embodiment comprises  
6 the combined administration of an anti-CD3 immunotoxin according  
7 to the invention and still other spergualin analogs, such as  
8 compounds disclosed in U.S. Patent No. 5,476,870 and EP 600,762,  
9 both incorporated by reference, e.g.,

10

11



12

13

14

compound (a)

15

16 i.e. 2-[[[4-[[3-(Amino)propyl]amino]butyl]amino]  
17 carbonyloxy]-N-[6-[(aminoiminomethyl)amino] hexyl]acetamide  
18 ("tresperimus") and

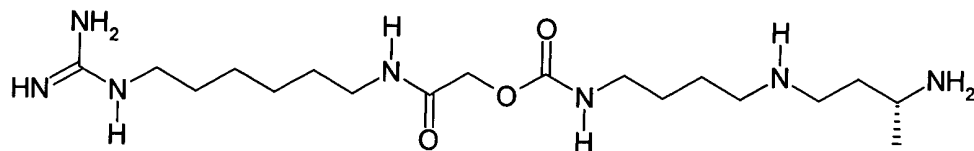
19

20 its pharmaceutically acceptable addition salts with a mineral or  
21 organic acid;

22

23 compounds disclosed in U.S. Patent No. 5,637,613 and EP  
24 669,316, both incorporated by reference, e.g.,

25



26

27

compound (b)

28

1 i.e. 2-[[[4-[[3(R)-(Amino)butyl]amino]butyl]amino  
2 carbonyloxy]-N-[6-[(aminoiminomethyl) amino]hexyl] acetamide tris  
3 (trifluoroacetate) and other pharmaceutically acceptable salts  
4 thereof. Pharmaceutically acceptable salts of the above  
5 compounds include salts with a mineral acid or an organic acid,  
6 including (with respect to mineral acids) hydrochloric,  
7 hydrobromic, sulfuric and phosphoric acid, and (with respect to  
8 organic acids) fumaric, maleic, methanesulfonic, oxalic and  
9 citric;

10

11 compounds disclosed in U.S. Patent No. 5,733,928 and EP  
12 743,300, both incorporated by reference;

13

14 compounds disclosed in U.S. Patent No. 5,883,132 and EP  
15 755,380, both incorporated by reference; and

16

17 compounds disclosed in USP 5,505,715 (e.g., col. 4, l. 44 -  
18 col. 5 , l. 45), incorporated by reference.

19

20 By "combined administration" is meant treatment of the organ  
21 transplant recipient with both an anti-CD3 immunotoxin of the  
22 invention and the spergualin derivative or analog.

23

24 Administration of the immunotoxin and the spergualin  
25 derivative or analog need not be carried out simultaneously, but  
26 rather may be separated in time. Typically, however, the course  
27 of administration of the immunotoxin and the spergualin related  
28 compound will be overlapping to at least some extent.

29

30 The total dose of the anti-CD3 immunotoxin is preferably  
31 given over 2-3 injections, the first dose preceding the

1 transplant by the maximal time practicable, with subsequent  
2 injections spaced by intervals of, for example, about 24 hours.

3

4 The immunotoxin is preferably administered prior to  
5 transplant and at the time of and/or following transplant.

6

7 In allotransplantation, administration of the anti-CD3  
8 immunotoxin preferably precedes transplant surgery by about 2-6  
9 hours, whereas for xenotransplantation or living related  
10 allotransplantation, the first anti-CD3 immunotoxin injection may  
11 precede transplantation by as much as one week, see for example,  
12 Knechtle, S. J., et al. (1997) *FN18-CRM9 immunotoxin promotes*  
13 *tolerance in primate renal allografts*. Transplantation 63: 1.

14

15 In a tolerance induction regimen, the immunotoxin treatment  
16 is preferably curtailed no later than about 14 days, and  
17 preferably on about day 7, or on day 5, or even on day 3,  
18 post-transplant.

19

20 The spergualin derivative or analog may be administered  
21 prior to transplant, at the time of transplant, and/or following  
22 transplant. The length of treatment either before or after  
23 transplant may vary.

24

25 In a tolerance induction regimen, the treatment with  
26 spergualin derivative or analog compound is preferably withdrawn  
27 not later than about 120 days following transplant, and more  
28 preferably after about 60 days post-transplant, and more  
29 preferably after about 30 days, and even more preferably not  
30 later than 14, or even about 10 days, post-transplant.

1        Thus, the term "combined administration" includes within its  
2 scope a treatment regimen wherein, for example, one or more doses  
3 of immunotoxin is/are administered prior to the transplant,  
4 followed by one or more doses commencing at around the time of  
5 transplant; together with administration of the spergualin  
6 derivative or analog also prior to and/or at the time of  
7 transplant, and typically continuing after transplant.

8

9        Corticosteroids such as methylprednisolone may be  
10 incorporated into the combined administration regimen. For  
11 example, steroid administration may commence prior to transplant,  
12 and may continue with one or more doses thereafter.

13

14        The anti-CD3 immunotoxin of the invention is preferably  
15 provided in a dose sufficient to reduce the T-cell number in a  
16 patient by 2-3 logs.

17

18        A total effective dosage to reduce the T-cell number in a  
19 patient by 2-3 logs in accordance herewith may be between about  
20 50 µg/kg and about 10 mg/kg body weight of the subject, and more  
21 preferably between about 0.1 mg/kg and 1 mg/kg.

22

23        A dosage regimen for an induction treatment with the  
24 spergualin derivative or analog may be between 1 and 10 mg/kg/day  
25 for 0-30 days, optimally, for example about 2.5 mg/kg/day for 15  
26 days.

27

28        Additional steroids may be administered at the time of the  
29 anti-CD3 immunotoxin injections, for example as a decreasing  
30 regimen of methylprednisone, such as 7 mg/kg on the day of the  
31 transplant surgery, 3.5 mg/kg at +24 hours, and 0.35 mg/kg at +48  
32 hours. Alternatively, the steroid dosage may be held constant,

1 for example treatment with 40 mg/kg of prednisone at the time of  
2 immunotoxin injection. It is understood that the exact amount  
3 and choice of steroid can vary, consistent with standard  
4 clinical practice.

5

6 In a preferred embodiment of the combination therapy of the  
7 invention, the immunotoxin of the combined therapy is scFv  
8 (UCHT-1)-PE38, and is in particular an immunotoxin having SEQ.  
9 ID. No:1.

10

11 Said scFv(UCHT-1)-PE38 is preferably co-administered with  
12 15-deoxyspergualine, and especially, (-)-15-deoxyspergualine.

13

14 In another aspect, said scFv(UCHT-1)-PE38 is co-  
15 administered with the abovementioned compound (a).

16

17 In a still further embodiment, said scFv(UCHT-1)-PE38 is  
18 co-administered with the abovementioned compound (b).

19

20 In the practice of the above combination therapy and the  
21 other methods of this invention in the context of  
22 xenotransplantation, and especially where the transplant  
23 recipient is human, the donor cells, tissues or organs are  
24 preferably porcine, and are most preferably recruited from  
25 transgenic, e.g., human DAF expressing, pigs.

26

27 In another embodiment of the methods of the invention, the  
28 immunotoxins can be administered in vivo to a bone marrow  
29 recipient for prophylaxis or treatment of host-versus-graft

1 disease through killing of host (i.e. bone marrow transplant  
2 recipient) T cells. Marrow transplants become necessary in the  
3 treatment of certain diseases, such as leukemia, aplastic anemia  
4 or certain genetic disorders, in which the patient's own marrow  
5 is severely flawed or where total body irradiation or  
6 chemotherapy have destroyed the patient's hematopoietic system.  
7 Absent reconstitution of the hematopoietic system by bone marrow  
8 transplantation, the patient becomes severely immunodepressed and  
9 susceptible to infection.

10

11       Stable engraftment of donor allogeneic bone marrow depends  
12 in large part on MHC matching between donor and recipient. In  
13 general, mismatching only to the extent of one or two antigens is  
14 tolerable in bone marrow transplantation because of rejection of  
15 the disparate bone marrow graft by recipient T cells. (Also,  
16 graft versus host disease, discussed below, is very severe when  
17 there are greater disparities.) In addition, even minor  
18 mismatching conventionally necessitates conditioning of the  
19 recipient by lethal or sublethal doses of total body irradiation  
20 or total lymphoid irradiation to deplete recipient T-cells. This  
21 requirement for irradiation of the bone marrow transplant patient  
22 which renders the patient totally or nearly immunoincompetent  
23 poses a significant limitation on clinical application of bone  
24 marrow transplantation to a variety of disease conditions in  
25 which it is potentially useful, including solid organ or cellular  
26 transplantation, sickle cell anemia, thalassemia and aplastic  
27 anemia.

28

29       The present invention addresses this problem by providing a  
30 directed means of killing recipient T cells in the absence of  
31 radiation.

32



1        Thus this invention provides in another of its aspects, a  
2 method for conditioning a bone marrow transplant patient prior  
3 to engraftment in the patient of donor bone marrow and/or stem-  
4 cell enriched peripheral blood cells, comprising administration  
5 of a T-cell depleting effective amount of immunotoxin to the  
6 patient. The immunotoxin effects reductions in the T cell  
7 population in the patient and thereby exerts a prophylaxis  
8 against host (i.e. the patient's) rejection of the donor bone  
9 marrow graft. Methods of obtaining donor compositions enriched  
10 for hematopoietic stem cells are disclosed in U.S. 5,814,440,  
11 U.S. 5,681,559, U.S. 5,677,136, and U.S. 5,061,620, all  
12 incorporated by reference.

13  
14        Graft-versus-host disease (GVHD), in particular, is a  
15 sometimes fatal, often debilitating complication of allogeneic  
16 bone marrow transplant which is mediated primarily, if not  
17 exclusively, by T lymphocytes. GVHD is caused by donor T cells  
18 which are acquired in the graft by the bone marrow recipient and  
19 which develop an immune response against the host. GVHD  
20 typically results from incomplete immunologic matching of donor  
21 and recipient Human leukocyte antigens (HLA).

22  
23        Accordingly, this invention also contemplates a method of  
24 prophylaxis or treatment of GVHD in a bone marrow transplant  
25 patient, comprising administration of an immunotoxin of the  
26 invention to the patient during the early post-transplant  
27 period, or when symptoms of GVHD become manifest, in an amount  
28 sufficient to effect reductions in levels of T cells in the host  
29 (i.e. patient), including both donor and host T cells. The  
30 early depletion of donor and host T-cells also facilitates the  
31 development of allogeneic chimerism; that is, the T cells which  
32 are given space to mature following host T-cell ablation by  
33 immunotoxin are rendered tolerant of both donor and host

1 antigens and do not participate in graft versus host rejection.  
2 By "early post-transplant period" is meant a period of one or  
3 more days up to about two weeks following bone marrow  
4 transplantation.

5  
6 In a further embodiment, the anti-CD3 immunotoxin of the  
7 invention can be administered to a patient in need thereof to  
8 treat still other T-cell mediated pathologies, such as T-cell  
9 leukemias and lymphomas. As mentioned above, clinical treatment  
10 of T-cell leukemias and lymphomas typically relies on whole  
11 body irradiation to indiscriminately kill lymphoid cells of a  
12 patient, followed by bone marrow replacement. An immunotoxin of  
13 the invention administered to a patient suffering from  
14 leukemia/lymphoma can replace whole body radiation with a  
15 selective means of eliminating T-cells.

16  
17 In additional aspects of the invention, the immunotoxins of  
18 the invention may also be administered to a patient in vivo to  
19 treat T-cell-mediated autoimmune disease, such as systemic lupus  
20 erythematosus (SLE), type I diabetes, rheumatoid arthritis (RA),  
21 myasthenia gravis, and multiple sclerosis, by ablating  
22 populations of T cells in the patient.

23  
24 The immunotoxins can also be administered to a subject  
25 afflicted with an infectious disease of the immune system, such  
26 as acquired immune deficiency syndrome (AIDS), in an amount  
27 sufficient to deplete the patient of infected T-cells and  
28 thereby inhibit replication of HIV-1 in the patient.

29  
30 Additionally, the anti-CD3 immunotoxin can be administered  
31 to patients to treat conditions or diseases in instances in  
32 which chronic immunosuppression is not acceptable, e.g., by  
33 facilitating islet or hepatocyte transplants in patients with

1 diabetes or metabolic diseases, respectively. Diseases and  
2 susceptibilities correctable with hepatocyte transplants include  
3 hemophilia,  $\alpha$ 1-antitrypsin insufficiencies, and  
4 hyperbilirubinemias.

5  
6 In the above methods of the invention, the patient is  
7 preferably human and the donor may be allogeneic (i.e. human) or  
8 xenogeneic (e.g., swine). The transplant may be an unmodified or  
9 modified organ, tissue or cell transplant, e.g., heart, lung,  
10 combined heart-lung, trachea, liver, kidney, pancreas, Islet  
11 cell, bowel (e.g., small bowel), skin, muscles or limb, bone  
12 marrow, oesophagus, cornea or nervous tissue transplant.

13  
14 For in vivo applications, the immunotoxin will be  
15 administered to the patient in an amount effective to kill at  
16 least a portion of the targeted population of CD3-bearing cells  
17 (i.e. T-cells).

18  
19 In general, an effective amount of immunotoxin will deplete  
20 a targeted population of T cells, i.e. in the lymph system  
21 and/or peripheral blood, by 1 or more logs, and more preferably  
22 by at least about 2 logs, and even more preferably by at least  
23 2-3 logs. The most effective mode of administration and dosage  
24 regimen depends on the severity and course of the disease, the  
25 subject's health and response to treatment and the judgment of  
26 the treating physician. Thus the dosages of the molecules  
27 should be titrated to the individual subject.

28  
29 Preferably, in the treatment or prophylaxis of GVHD  
30 accompanying bone marrow transplantation, the immunotoxin is  
31 administered to the bone marrow transplant recipient in an  
32 amount sufficient to reduce the total T-cell population (i.e.  
33 donor plus recipient T cells) present in the patient blood and

1 lymph nodes immediately following bone marrow transplantation by  
2 at least about 50% and more preferably at least about 80%, and  
3 even more preferably at least about 95% (e.g., 99%), i.e. by at  
4 least 2 logs (e.g., by 2-3 logs).

5  
6 A suitable dosing regimen for a bone marrow recipient, to  
7 treat or prevent host versus graft disease and/or GVHD, may  
8 comprise administration of immunotoxin immediately prior to,  
9 and/or immediately following bone marrow transplantation on each  
10 alternating day over the course of six days after transplant, to  
11 bring the total dose to about 10-500 µg/kg, and more preferably  
12 200-300 µg/kg.

13  
14 For treatment of leukemia/lymphoma, the immunotoxin is  
15 administered in an amount sufficient to reduce the T-cell  
16 population at the time of administration by at least about 50%,  
17 and more preferably at least about 80%, and more preferably at  
18 least about 95% (e.g., 99%), i.e. by at least 2 logs (e.g., by  
19 at least 2-3 logs).

20  
21 The levels of CD3-bearing cells, and in particular, of T  
22 cells, in the patient's bone marrow, blood or lymphoid tissues,  
23 can be assayed by FACS analysis.

24  
25 The effectiveness of immunotoxin treatment in depleting T-  
26 cells from the peripheral blood and lymphoid organs can be  
27 determined by comparing T-cell counts in blood samples and from  
28 macerated lymphoid tissue taken from the subject before and after  
29 immunotoxin treatment. Depletion of T-cells can be followed by  
30 flow cytometry as described by Neville et al., 1996, *J.*  
31 *Immunother.* 19:95-92.

1 Depletion of T-cell numbers by 2 logs, by a chemically  
2 conjugated immunotoxin comprised of an anti-rhesus CD3 monoclonal  
3 antibody conjugated to a cell binding domain-deleted form of  
4 diphtheria toxin, has been shown to be associated with  
5 transplantation tolerance to renal allografts in rhesus monkeys  
6 (Thomas et al., 1997, *Transplantation* 64:124-135; Knechtle et  
7 al., 1997, *Transplantation* 63:1-6).  
8

9 In general, a total effective dosage to reduce the T-cell  
10 number in a patient by 2-3 logs in accordance herewith can best  
11 be described as between about 50 µg/kg and about 10 mg/kg (e.g.,  
12 between about 50 µg/kg and 5 mg/kg) body weight of the subject,  
13 and more preferably between about 0.1 mg/kg and 1 mg/kg.  
14

15 The patient may be treated on a daily basis in single or  
16 multiple administrations.  
17

18 The immunotoxin composition may also be administered on a  
19 per month basis (or at such weekly intervals as may be  
20 appropriate), also in either single or multiple administrations.  
21

22 It is envisaged that, in the course of the disease state,  
23 the dosage and timing of administration may vary. Initial  
24 administrations of the composition may be at higher dosages  
25 within the above ranges, and administered more frequently than  
26 administrations later in the treatment of the disease.  
27

28 For example, the polypeptide, scFv(UCHT-1)-PE38 of Example  
29 1, may be administered to a kidney transplant patient starting  
30 just prior to transplantation and continuing, post-transplant,  
31 over the course of a week in daily or alternate day dosing, at a  
32 dose of about 0.3 -10 mg per week of polypeptide in the average

1 patient (70 kg). After the first week post-transplant, the  
2 treatment regimen may be reduced to alternating weeks, with  
3 dosages ranging from 0.1 mg to 1 mg of polypeptide per week in  
4 the average patient. It is expected, however, that immunotoxin  
5 treatment shall be curtailed at five weeks after transplant, and  
6 more typically at three weeks, or even at one week post-  
7 transplant.

#### 8 9 Ex Vivo Applications

10  
11 It is also within the scope of the present invention to  
12 utilize the immunotoxins for purposes of ex vivo depletion of T  
13 cells from isolated cell populations removed from the body.

14  
15 In one aspect, the immunotoxins can be used in a method for  
16 prophylaxis of organ transplant rejection, wherein the method  
17 comprises perfusing the donor organ (e.g., heart, lung, kidney,  
18 liver) prior to transplant into the recipient with a composition  
19 comprising a T-cell depleting effective amount of immunotoxin, in  
20 order to purge the organ of sequestered donor T-cells.

21  
22 In another embodiment of the invention, the immunotoxins can  
23 be utilized ex vivo in an autologous therapy to treat T cell  
24 leukemia/lymphoma or other T-cell mediated diseases or conditions  
25 by purging patient cell populations (e.g., bone marrow) of  
26 cancerous or otherwise affected T-cells with immunotoxin, and  
27 reinfusing the T-cell-depleted cell population into the patient.

28  
29 In particular, such a method of treatment comprises:

30 (a) recruiting from the patient a cell population  
31 comprising CD3-bearing cells (e.g., bone marrow);

32 (b) treating the cell population with a T-cell depleting  
33 effective amount of immunotoxin; and

1 (c) infusing the treated cell population into the patient  
2 (e.g., into the blood).  
3

4 A still further application of such an autologous therapy  
5 comprises a method of treating a subject infected with HIV,  
6 comprising the steps of:  
7

8 (a) isolating a cell population from the patient comprising  
9 T cells infected with HIV.

10 (b) treating the isolated cell population with a T-cell-  
11 depleting effective amount of immunotoxin; and

12 (c) reintroducing the treated cell population into the  
13 patient.  
14

15 According to still another embodiment of the invention, the  
16 immunotoxins can be utilized ex vivo for purposes of effecting T  
17 cell depletion from a donor cell population as a prophylaxis  
18 against graft versus host disease, and induction of tolerance,  
19 in a patient to undergo a bone marrow transplant. Such a method  
20 comprises the steps of:

21 (a) providing a cell composition comprising isolated bone  
22 marrow and/or stem cell-enriched peripheral blood cells of a  
23 suitable donor (i.e. an allogeneic donor having appropriate MHC,  
24 HLA-matching);

25 (b) treating the cell composition with an effective amount  
26 of immunotoxin to form an inoculum at least partially depleted  
27 of viable CD3-bearing cells (i.e. T-cells); and

28 (c) introducing the treated inoculum into the patient.  
29

30 By virtue of T-cell depletion from the donor inoculum, the  
31 donor T cells which mature following engraftment are rendered  
32 immunologically tolerant of the host and will not initiate graft  
33 versus host rejection.

1        Advantageously, for purposes of the above-described ex vivo  
2        therapies, the immunotoxin can be provided in a therapeutic  
3        concentration far in excess of levels which could be accomplished  
4        or tolerated in vivo.

5  
6        For example, the immunotoxin may be incubated with CD3-  
7        expressing cells in culture at a concentration of about 0.5 to  
8        50,000 ng/ml in order to kill CD3-bearing cells in said culture.

9  
10       Thus, it has been found that incubation of human cytokine-  
11       mobilized peripheral blood leukocytes (CMPBL,  $5 \times 10^6$ /ml) in  
12       culture medium for one hour at 25°C. with 0.005 to 50 µg/ml of  
13       the immunotoxin prepared in Example 1, results in depletion of  
14       the number of CD3<sup>+</sup> cells by about 2.5 logs, and reduces PHA-  
15       induced proliferation to background levels as measured by <sup>3</sup>H-  
16       thymidine uptake.

17  
18       In a further aspect, the above ex vivo therapeutic methods  
19       can be combined with in vivo administration of immunotoxin, to  
20       provide improved methods of treating or preventing rejection in  
21       bone marrow transplant patients, and for achieving immunological  
22       tolerance.

23  
24       For example, a method comprising both in vivo and ex vivo  
25       administration of an immunotoxin of the invention for the  
26       prophylaxis and/or treatment of host versus graft disease and/or  
27       graft versus host disease in a patient to undergo a bone marrow  
28       transplant comprises the steps of:

29       (a) reducing the levels of viable CD3-bearing cells (i.e.  
30       T cells) in the patient (i.e. from the patient's peripheral  
31       blood or lymph system);

32       (b) providing an inoculum comprising hematopoietic cells  
33       (i.e. bone marrow and/or stem cell-enriched peripheral blood



1 cells) of a suitable donor treated with a T-cell depleting  
2 effective amount of immunotoxin; and

3 (c) introducing the inoculum into the patient, and  
4 thereafter optionally administering immunotoxin to the patient  
5 to further deplete donor and patient T cells.

6  
7 Step (a), i.e. depletion of patient T cells can be carried  
8 out by in vivo administration of immunotoxin to the patient  
9 and/or by an autologous therapy comprising ex vivo treatment of  
10 isolated patient bone marrow or peripheral blood with  
11 immunotoxin, as previously described.

12  
13 The in vivo and ex vivo methods of the invention as  
14 described above are suitable for the treatment of diseases  
15 curable or treatable by bone marrow transplantation, including  
16 leukemias, such as acute lymphoblastic leukemia (ALL), acute  
17 nonlymphoblastic leukemia (ANLL), acute myelocytic leukemia  
18 (AML), and chronic myelocytic leukemia (CML), cutaneous T-cell  
19 lymphoma, severe combined immunodeficiency syndromes (SCID),  
20 osteoporosis, aplastic anemia, Gaucher's disease, thalassemia,  
21 mycosis fungoides (MF), Sezary syndrome (SS), and other  
22 congenital or genetically-determined hematopoietic  
23 abnormalities.

24  
25 In particular, it is also within the scope of this invention  
26 to utilize the immunotoxins as agents to induce donor-specific  
27 and antigen-specific tolerance in connection with allogeneic or  
28 xenogeneic cell therapy or tissue or organ transplantation.  
29 Thus, the immunotoxin can be administered as part of a  
30 conditioning regimen to induce immunological tolerance in the  
31 patient to the donor cells, tissue or organ, e.g., heart, lung,  
32 combined heart-lung, trachea, liver, kidney, pancreas, Islet  
33

1 cell, bowel (e.g., small bowel), skin, muscles or limb, bone  
2 marrow, oesophagus, cornea or nervous tissue.

3  
4       Systemic donor-specific transplantation tolerance has been  
5 transiently achieved in MHC-mismatched animal models as well as  
6 in humans through chimerism as a result of total lymphoid  
7 irradiation of a recipient followed by bone marrow  
8 transplantation with donor cells. The reconstituted animals  
9 exhibit stable mixed multilineage chimerism in their peripheral  
10 blood, containing both donor and recipient cells of all  
11 lymphohematopoietic lineages, including T cells, B cells,  
12 natural killer cells, macrophages, erythrocytes and platelets.  
13 Furthermore, the mixed allogeneic chimeras display donor-  
14 specific tolerance to donor-type skin grafts, while they readily  
15 reject third-party grafts. Donor-specific tolerance is also  
16 confirmed by in vitro assays in which lymphocytes obtained from  
17 the chimeras are shown to have diminished proliferative and  
18 cytotoxic activities against allogeneic donor cells, but retain  
19 normal immune reactivity against third-party cells.

20  
21       Thus the present invention further contemplates a method of  
22 conditioning a patient to be transplanted with donor cells, or a  
23 tissue or organ. The method comprises the steps of:

24       (a) reducing levels of viable CD3-bearing (i.e. T cells)  
25 in the patient (i.e. in the peripheral blood or lymph system of  
26 the patient);

27       (b) providing an inoculum comprising isolated  
28 hematopoietic cells (i.e. bone marrow and/or stem-cell enriched  
29 peripheral blood cells) of the donor treated with a T-cell  
30 depleting effective amount of immunotoxin;

31       (c) introducing the inoculum into the patient; and  
32 thereafter,

1 (d) transplanting the donor cells, tissue or organ into the  
2 patient.

3  
4 The above method is preferably carried out in the absence  
5 of total body irradiation or total lymphoid irradiation, and  
6 most preferably, in the absence of any radiation.

7  
8 6. Compositions Comprising Immunotoxin  
9

10 The recombinant immunotoxin polypeptide of the invention  
11 can be administered as an unmodified polypeptide or its pharma-  
12 ceutically acceptable salt, in a pharmaceutically acceptable  
13 carrier.

14  
15 As used herein the term "pharmaceutically acceptable salt"  
16 refers to salts prepared from pharmaceutically acceptable non-  
17 toxic acids to form acid addition salts of an amino group of the  
18 polypeptide chain, or from pharmaceutically acceptable non-toxic  
19 bases to form basic salts of a carboxyl group of the polypeptide  
20 chain. Such salts may be formed as internal salts and/or as  
21 salts of the amino or carboxylic acid terminus of the  
22 polypeptide of the invention.

23  
24 Suitable pharmaceutically acceptable acid addition salts  
25 are those of pharmaceutically acceptable, non-toxic organic  
26 acids, polymeric acids, or inorganic acids.

27  
28 Examples of suitable organic acids comprise acetic,  
29 ascorbic, benzoic, benzensulfonic, citric, ethanesulfonic,  
30 fumaric, gluconic, glutamic, hydrobromic, hydrochloric,  
31 isethionic, lactic, maleic, malic, mandelic, methanesulfonic,  
32 mucic, nitric, oxalic, pamoic, pantothenic, phosphoric,

1 salicylic, succinic, sulfuric, tartaric, p-toluenesulfonic,  
2 etc., as well as polymeric acids such as tannic acid or  
3 carboxymethyl cellulose. Suitable inorganic acids include  
4 mineral acids such as hydrochloric, hydrobromic, sulfuric,  
5 phosphoric, nitric acid, and the like.

6  
7 Examples of suitable inorganic bases for forming salts of a  
8 carboxyl group include the alkali metal salts such as sodium,  
9 potassium and lithium salts; the alkaline earth salts such as  
10 for example calcium, barium and magnesium salts; and ammonium,  
11 copper, ferrous, ferric, zinc, manganous, aluminum, manganic  
12 salts, and the like. Preferred are the ammonium, calcium,  
13 magnesium, potassium, and sodium salts.

14  
15 Examples of pharmaceutically acceptable organic bases  
16 suitable for forming salts of a carboxyl group include organic  
17 amines, such as, for example, trimethylamine, triethylamine,  
18 tri(n-propyl)amine, dicyclohexylamine, beta(dimethylamino)-  
19 ethanol, tris(hydroxymethyl)aminomethane, triethanolamine,  
20 beta-(diethylamino)ethanol, arginine, lysine, histidien,  
21 N-ethylpiperidine, hydrabamine, choline, betaine,  
22 ethylenediamine, glucosamine, methylglucamine, theobromine,  
23 purines, piperazines, piperidines, caffeine, procaine, and the  
24 like.

25  
26 Acid addition salts of the polypeptides may be prepared in  
27 the usual manner by contacting the polypeptide with one or more  
28 equivalents of the desired inorganic or organic acid, such as,  
29 for example, hydrochloric acid.

30  
31 Salts of carboxyl groups of the peptide may be convention-  
32 ally prepared by contacting the peptide with one or more  
33 equivalents of a desired base such as, for example, a metallic

1 hydroxide base e.g., sodium hydroxide; a metal carbonate or  
2 bicarbonate base such as, for example, sodium carbonate or  
3 sodium bicarbonate; or an amine base such as for example  
4 triethylamine, triethanolamine, and the like.

5  
6 For either in vivo or ex vivo applications, the  
7 pharmaceutical compositions of the invention comprise a carrier  
8 which is preferably a sterile, pyrogen-free, parenterally  
9 acceptable liquid.

10  
11 Water, physiological saline, aqueous dextrose, and glycols  
12 are preferred liquid carriers, particularly (when isotonic) for  
13 injectable solutions, or for ex vivo uses.

14  
15 Compositions comprising the immunotoxin or its salt can be  
16 administered systemically, i.e. parenterally (e.g.,  
17 intramuscularly, intravenously, subcutaneously or  
18 intradermally), or by intraperitoneal administration.

19  
20 Compositions particularly useful for parenteral  
21 administration, such as intravenous administration or  
22 administration into a body cavity or lumen of an organ will  
23 commonly comprise a solution of the fusion protein dissolved in  
24 a pharmaceutically acceptable carrier, preferably an aqueous  
25 carrier such as buffered saline or the like. These compositions  
26 are sterile and generally free of undesirable matter. These  
27 compositions may be sterilized by conventional, well-known  
28 sterilization techniques. The compositions may also contain  
29 pharmaceutically acceptable auxiliary substances as required to  
30 approximate physiological conditions such as pH adjusting and  
31 buffering agents, toxicity adjusting agents and the like, for  
32 example, sodium acetate, sodium chloride, potassium chloride,  
33 calcium chloride, sodium lactate and the like. The

1 concentration of immunotoxin protein in these formulations can  
2 vary widely, and will be selected primarily based on fluid  
3 volumes, viscosities, body weight and the like in accordance  
4 with the particular mode of administration selected and the  
5 patient's needs. Actual methods for preparing parenterally  
6 administrable compositions will be known or apparent to those  
7 skilled in the art and are described in more detail in such  
8 publications as Remington's Pharmaceutical Science, 15<sup>th</sup> ed.,  
9 Mack Publishing Company, Easton, Pa. (1980).

10  
11       Pharmaceutical compositions comprising the immunotoxins or  
12 their salts can also be used for oral, topical, or local  
13 administration, such as by aerosol or transdermally.

14  
15       Unit dosage forms suitable for oral administration include  
16 powder, tablets, pills, capsules and lozenges. It is recognized  
17 that the polypeptides, when administered orally, must be  
18 protected from digestion, such as by complexing the protein with  
19 a composition to render it resistant to acidic and enzymatic  
20 hydrolysis or by packaging the protein in an appropriately  
21 resistant carrier such as a liposome. Various means of  
22 protecting proteins from digestion are known in the art.

23  
24       Examples of the topical dosage form include sprays,  
25 ophthalmic solutions, nasal solutions and ointments.

26  
27       For example, a spray can be manufactured by dissolving the  
28 peptide in an appropriate solvent and putting it in a spray to  
29 serve as an aerosol for commonly employed inhalation therapy.  
30 An ophthalmic or nasal solution can be manufactured by dissolving  
31 the active ingredient peptide in distilled water, adding any  
32 auxiliary agent required, such as a buffer, isotonicizing agent,

1 thickener, preservative, stabilizer, surfactant, antiseptic,  
2 etc., and adjusting the mixture to pH 4 to 9.

3  
4 Ointments can also be prepared, e.g., by preparing a  
5 composition from a polymer solution, such as 2% aqueous  
6 carboxyvinyl polymer, and a base, such as 2% sodium hydroxide,  
7 mixing to obtain a gel, and mixing with the gel an amount of  
8 purified fusion polypeptide.

9  
10 The composition may be a lyophilizate prepared by methods  
11 well known in the art.

12  
13 In the practice of the in vivo methods of the present  
14 invention, a therapeutically effective amount of a recombinant  
15 immunotoxin polypeptide, a pharmaceutically acceptable salt  
16 thereof, or a pharmaceutical composition containing same, as  
17 described above, is administered to a patient in need thereof.

18  
19 The following exemplification is presented to illustrate  
20 the present invention and provide assistance to one of ordinary  
21 skill in making and using the same, and is not intended to be  
22 limitative of the scope of the invention.

23  
24  
25 Example 1. Preparation of scFv(UCHT-1)-PE38.

26 (a) Cloning of UCHT-1 antibody variable regions from  
27 hybridoma cells.

28  
29 The genes encoding the Fv region of murine anti-human CD3  
30 are amplified by RT-PCR from UCHT-1 hybridoma RNA (Beverley and  
31 Callard, 1981) using oligonucleotide primers based upon the  
32 published sequence of UCHT-1 scFv (Shalaby et al. (1992), *supra*,  
33 and upon consensus primers described for cloning antibody

1 variable regions (Orlandi et al. (1989) PNAS 86: 3833-3387), as  
2 listed in Table I.

3  
4 Oligos IM34A and IM34B are used to amplify the V<sub>L</sub> region,  
5 and IM-61 and IM-34C are used to amplify the V<sub>H</sub> fragment. The  
6 two amplified fragments are then subcloned into E. coli plasmid  
7 vectors (TA Vector, Invitrogen) and their DNA sequences  
8 determined.

9  
10 After determining the cloned DNA sequences, the two  
11 molecules are combined into a single pUC18-based plasmid by  
12 cutting pUC18 and the subcloned PCR-fragments at the appropriate  
13 restriction sites and ligating them together with T4 DNA ligase.  
14 This plasmid, containing V<sub>L</sub> followed by a polylinker which is in  
15 turn followed by V<sub>H</sub>, is cut with XbaI plus SalI. A linker  
16 comprised of the two annealed oligos, IM-24A and IM24B, designed  
17 to contain complementary ends for these two sites, is inserted  
18 between the XbaI and SalI sites. The resultant clone, 'CloneB',  
19 encodes a single chain immunotoxin with a linker different than  
20 that described in SEQ. ID NO:1. The replacement of this linker  
21 with the (Gly<sub>3</sub>Ser)<sub>4</sub> (SEQ. ID. NO:5) linker used in scFv(UCHT-  
22 1)PE38 is described below. However, it was first necessary to  
23 investigate two changes in the variable region sequences which  
24 are observed relative to the sequence of the clone Fv fragment  
25 reported in Shalaby, et al., supra:

26  
27 (1) a change of A to C at nucleotide position 208 in the  
28 heavy chain sequence (V<sub>H</sub>). This is likely to reflect an error by  
29 Shalaby et al. (1992), supra, since the amino acid (Leu)  
30 reportedly encoded at this position, does not correlate with the  
31 nucleotide sequence in the paper but does correlate with the  
32 sequence of the presently obtained clone; and

33



1           (2) a change of Phe to Ser at amino acid residue 98. This  
2 appears to be a PCR-induced error, and this point mutation in V<sub>L</sub>  
3 is corrected using a standard 4-way PCR reaction in which the  
4 desired nucleotide change is incorporated using complementary  
5 oligos VL2 and VL3. Flanking oligos, VL1 on the 5' side and VH4  
6 on the 3' side, stabilize the change, as described below.

7  
8 a1. Correction of point mutation in V<sub>L</sub>

9           PCR reactions using pUC18/UCHT-1 'Clone B' as template are  
10 set up with oligo pairs VL1 and VL2 or VL3 and VH4. The two  
11 distinct PCR products are separated by gel electrophoresis, their  
12 complementary ends are annealed, and a second PCR reaction in  
13 which VL1 and VH4 are used to join these two fragments is  
14 performed using the previously annealed products as a template.

15  
16 a2. Relacement of linker from 'Clone B'

17           The linker separating V<sub>L</sub> and V<sub>H</sub> is changed to a linker  
18 containing the sequence (Gly<sub>3</sub> Ser)<sub>4</sub> (SEQ. ID. NO:5) by two  
19 sequential PCR reactions, using the plasmid with the point  
20 mutation corrected as template. The 5' primer for both  
21 sequential reactions is complementary to the vector sequences  
22 (M13R; New England Biolabs). The 3' primer for the first PCR  
23 reaction is VL6, and the 3' primer for the second reaction is  
24 VL8. VL6 and VL8 are complementary to the coding strand; the  
25 BstXI site in VL8 occurs towards the N-terminus of the V<sub>H</sub>  
26 fragment of UCHT-1. The PCR product resulting from this second  
27 PCR reaction encodes the COOH-terminal end of V<sub>L</sub>, the new linker,  
28 and the N-terminus of V<sub>H</sub> (to just beyond the BSTXI site).

29  
30           The PCR product from this second PCR reaction is further  
31 extended in a third PCR reaction to add the N-terminal region of  
32 V<sub>L</sub>. This reaction uses the second PCR product as the 3' primer  
33 and the M13R (New England Biolabs) primer within the vector as

1 the 5' primer. The template for this third PCR reaction is the  
2 puc18/UCHT-1 'Clone B' plasmid. To substitute the second linker  
3 for the first and to attach the PCR product to the remainder of  
4 the  $V_H$ , the PCR product from this third reaction is cut with  
5 BamHI which occurs at the junction of  $V_L$  and the vector and with  
6 BstXI which occurs within  $V_H$ . The puc18/UCHT-1 'Clone B' plasmid  
7 also is cut with BamHI and BstXI; the corresponding area was  
8 substituted with the new product.

TABLE I.

IM-34A:	5'-GCGGATCCGACATCCAGATGACCCAGACCACC-3' (SEQ. ID. NO:11) (BamHI site is underlined).
IM-34B:	5'-CCTCTAGAAGCCCGTTTGATTTCCAGCTTGGT-3' (SEQ. ID. NO:12) (XbaI site is underlined).
IM-34C:	5'-CCAAGCTTTCATGAGGAGACGGTGACCGTGGTCCC-3' (SEQ. ID. NO:13) (HindIII site is underlined).
IM-61:	Coding oligo used for cloning V <sub>H</sub> : 5'-CCGTCGACGAGGTGCAGCTCCAGCAGTCT-3' (SEQ. ID. NO:14) (SalI site is underlined)
IM-24A:	The coding oligo for the linker is: 5'-CTAGAGGAGGTAGTGGAGGCTCAGGAGTTCTGGAGGTAGTG-3' (SEQ. ID. NO:15) (partial XbaI and SalI sites are underlined)
IM-24B:	The corresponding non-coding oligo for the linker is: 5'-TCGACACTACCTCCAGAACCTCCTGAGCCTCCACTACCTCCT-3' (SEQ. ID. NO:16) (The corresponding partial SalI and XbaI sites are underlined.)
VL1:	5' end of V <sub>L</sub> at nt 102-124: 5'-CTGGTATCAACAGAAACCAGATC-3' (SEQ. ID. NO:17)
VL2:	3' primer with the correct T at nt #293: 5'-GGTGCCTCCAGCGAACGTCCACGGAAG-3' (SEQ. ID. NO:18)
VL3:	5' primer with correct T at nt 293: 5'-CTTCCGTGGACGTTTCGCTGGAGGCACC-3' (SEQ. ID. NO:19)
VH4:	non-coding primer: 5'-CTCTGCTTCACCCAGTTCATG-3' (SEQ. ID. NO:20)
VL6:	5'-GCCACCGCTGCCTCCACCTGATCCACCGCCACTACCGCCTCC AGCCCGTTTGATTTCCAGCTTGGT-3' (SEQ. ID. NO:21)
VL8:	5'-TCAGGTCCAGACTGCTGGAGCTGCACCTCAGATCCGCCACCGC TGCCTCCACCTGAT-3' (SEQ. ID. NO:22) (BstXI site is underlined)

1           (b) Cloning of PE38.

2           The cloning of PE38 is described by Benhar et al.,  
3   *Bioconjugate Chem.*, Vol. 5, No. 4 (1994), and see also USP  
4   #5,981,726 and USP 5,990,296, incorporated by reference.

5  
6           (c) Preparation of Immunotoxin Fusion.

7           The new scFv is cloned into the pET15b E. coli expression  
8   vector (Novagen). Sites are first added to the scFv using PCR to  
9   make this fragment compatible with the pET15b cloning vector and  
10   with the HindIII site from the P. exotoxin-containing plasmid,  
11   pRB391 (kind gift of I. Pastan). (Alternatively, the DNA sequence  
12   encoding the PE38 fragment can be reconstructed from the pJH8  
13   plasmid which is deposited in the ATCC as ATCC #67208 using  
14   standard PCR methods and appropriate oligonucleotide primers. In  
15   this method, the pJH8 plasmid would require mutagenesis by PCR to  
16   add the HindIII site and the connector sequence present in the  
17   pRB391 plasmid and as described in Benhar, et al., 1994, supra.  
18   In addition, removal of the 16 amino acids (365-380 of native  
19   PE) of domain Ib internal to the PE40 fragment can be  
20   accomplished by PCR, resulting in a plasmid which is functionally  
21   identical to the PE38 fragment of pRB391. Confirmation that the  
22   resulting plasmid is in the same translational frame can be  
23   obtained by DNA sequence analysis.)

24  
25           The amino-terminal residues Met and Ala, encoded by an NcoI  
26   restriction site, are added to facilitate expression from the  
27   plasmid.

28

1       The amino acid and nucleotide sequences of the product  
2       (containing Met-Ala at the N-terminus) are given in SEQ. ID  
3       NOS:1 and 2, respectively, and FIGURE 15. A schematic  
4       representation of the protein is shown in Figure 2.

5  
6       In SEQ. ID NO:1, V<sub>L</sub> comprises residues 3-111, the peptide  
7       linker occupies residues 112-127, V<sub>H</sub> comprises residues 128-249,  
8       the connector is located at residues 250-254 and truncated PE  
9       comprises residues 255-601.

10  
11       In SEQ. ID. NO:2, DNA sequence encoding the NcoI, HindIII,  
12       and the EcoRI restriction sites used for subcloning, and the  
13       flexible linker separating the V<sub>L</sub> from the V<sub>H</sub> domains, are  
14       marked. The 3'-untranslated region, containing the EcoRI site  
15       (gaattc), and the BamHI/BglIII sites, is deleted.

16  
17       Expression of scFv(UCHT-1)-PE38 in E. coli strain BLR(DE3)  
18       is found to yield a highly homogenous product (i.e. 95% purity  
19       or greater) comprising the alanine-led polypeptide having  
20       residues 2-601 of SEQ. ID NO:1.

21  
22       (d) Fermentation, refolding and purification of scFv(UCHT-  
23       1)-PE38.

24       A process for the production of recombinant scFv(UCHT-1)-  
25       PE38 is established at the 50L scale. PET15b is transformed into  
26       E. coli BLR(DE3) (Novagen, Inc.). A fed-batch system using a  
27       self-regulatory, pH-stat-glycerol feeding strategy is employed.  
28       Feeding starts exactly after the initial amount of carbon source  
29       is depleted and glycerol is automatically fed in a limited  
30       manner, controlled by the pH. This procedure avoids the  
31       detrimental effect of an excess of glycerol and also of complete  
32       carbon-source depletion.

33

1           The optimal medium contains:  $\text{KH}_2\text{PO}_4$  @ 6 g/L,  $\text{KCl}$  @ 0.6 g/L,  
2  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  @ 0.2 g/L, N-Z-Amine A @ 24.0g/L, Yeast extract @ 72  
3 g/L,  $\text{Fe(III)}$ -ammonium citrate @ 100 mg/L,  $\text{MnSO}_4 \times \text{H}_2\text{O}$  @ 12 mg/L  
4 and glycerol @ 10g/L. For optimal expression levels, a lactose  
5 pulse induction is needed at  $\text{OD}_{550}$  of 50. Using this approach, 4.3  
6 kg of wet cell pellet containing 1 kg inclusion bodies are  
7 harvested after 24 hours from the fermentation experiment run  
8 under the conditions described in Table II (below).

9  
10 **Table II. Fermentation Conditions**

Parameter	Conditions
Volume	50 liter
Mixing:	200 - 250 rpm
Aeration / pressure	lvvm / 1 bar
$\text{pO}_2$ - control	Manual adjustment
pH-control	$6.7 < x < 7.1$ alkaline: 2 N NaOH
Temperature	37°C
Inoculum	1.0 L of pre culture grown in LB to $\text{OD}_{550} = 1.8$
Induction	50 g/L D-Lactose at $\text{OD}_{550} = 52$
Harvest:	11 hours after induction

11  
12  
13           Expression levels of 25% of total cellular protein are  
14 reached after induction with an excess of D-Lactose at  $\text{OD}_{550}$  of  
15 50 as assessed by densitometry of SDS-PAGE gels. Using this  
16 approach a productivity of 86 g wet cell pellet (wcp) and 20 g  
17 inclusion bodies (IBs) per liter fermenter broth are measured. A  
18 product titer of 1.4 g/L is determined by SDS-PAGE and  
19 densitometric quantification of scFv(UCHT-1)-PE38.

20  
21           The scFv(UCHT-1)-PE38 fusion protein is then extracted and  
22 refolded according to the general method of Buchner et al.  
23 (1992), *supra*, modified as follows:

1           (1) Frozen bacterial pellets (65g), containing induced  
2 scFv(UCHT-1)-PE38 in the form of inclusion bodies, are thawed at  
3 room temperature and subsequently transferred into 250 ml  
4 bottles. 180 ml of TES(50 mM Tris-HCL, pH 7.4, 20 mM EDTA and 100  
5 mM NaCl in water) are added to the bottles and the pellets are  
6 thoroughly suspended using a Polytron tissue disrupter. Portions  
7 of the suspended cells (30 ml) are distributed to fresh 250 ml  
8 bottles and diluted to 180 ml per bottle with TES. 8ml of  
9 lysozyme solution (8mg/ml in TES) are added to each bottle, the  
10 pellets are resuspended, and the suspensions are incubated at  
11 room temperature for one hour.

12  
13           (2) 20 ml of 25% Triton-X100 are added to each bottle, and  
14 the mixtures are shaken well. The mixtures are incubated at room  
15 temperature for thirty minutes. The cell lysates are then  
16 centrifuged at 13,000 rpm for fifty minutes using a GSA rotor.

17  
18           (3) The pellets are resuspended in 180 ml of TE (50 mM  
19 Tris-HCl, pH 7.4, and 20 mM EDTA). The suspensions are  
20 homogenized using a Polytron tissue disrupter for two minutes.  
21 20 ml of 25% Triton -X100 are added to each bottle and the  
22 mixtures are shaken well. The mixtures are centrifuged at 13,000  
23 rpm for ten (10) minutes.

24  
25           (4) The detergent (Triton-x100) wash steps described in (b)  
26 are repeated three times to produce relatively pure inclusion  
27 bodies. The inclusion bodies are resuspended in 180 ml of TE,  
28 and are then centrifuged at 13,000 rpm for ten (10) minutes.

29  
30           (5) The TE rinse steps described in (3) are repeated three  
31 times. The inclusion bodies are pooled and frozen as pellets at  
32 -70°C.

1           (6) 42 ml of solubilization buffer containing 6M Guanidine-  
2 HCl (MW=95.53) with 0.1 M Tris -HCl, pH 8.0 and 2 mM EDTA, is  
3 added to pooled inclusion bodies. The inclusion bodies are  
4 suspended by pipette. The suspension is transferred to two 50ml  
5 centrifuge tubes. The contents are incubated at room temperature  
6 overnight, and centrifuged.

7  
8           (7) 100 mg batches of denatured inclusion body protein are  
9 processed by reduction and renaturation. Dithioerythritol (DTE)  
10 is added to 0.3 M and the mixture is incubated at room  
11 temperature for two hours prior to the rapid addition of this  
12 sample (100 mg denatured inclusion body protein) to 100 volumes  
13 of refolding buffer. The refolding buffer is prepared by  
14 combining 0.1M Tris, pH 8.0, 0.5 M L-arginine- HCl (FW 210.7 g),  
15 and 2mM EDTA, adjusted to pH 9.5 with 10N NaOH, and equilibrated  
16 to 8-10°C prior to the addition of oxidized glutathione (GSSG, MW  
17 612.6g) to 8 mM. The sample is allowed to refold at 10°C for 30-  
18 40 hours without agitation. The sample is concentrated in a  
19 biocentrator and dialyzed into 20 mM Tris-HCl, pH 7.4, 1mM EDTA  
20 and 100mM Urea.

21  
22           (8) Refolded immunotoxin is purified by two sequential  
23 rounds of anion exchange chromatography, the first using Fast-  
24 Flow Q (Pharmacia) with a salt step gradient elution, and the  
25 second, using a Q5 column (BioRad) followed by a salt gradient  
26 elution. The following buffers are used during column  
27 chromatography for step and linear gradient elutions:

28  
29           equilibration: 20 mM Tris-HCl, pH 7.4, 1mM EDTA  
30           wash: 20 mM Tris-HCl, pH 7.4, 1mM EDTA, 0.08 M NaCl  
31           elution: 20 mM Tris-HCl, pH 7.4, 1mM EDTA, 0.28 M NaCl



1       Figure 3A shows a typical Fast Flow Q column purification  
2 profile. The eluted peak is then diluted 5-fold with  
3 equilibration buffer and applied to the Q5 column in the  
4 subsequent purification step. Figure 3B shows a typical Q5  
5 column profile.

6  
7       A single peak is recovered from the second anion-exchange  
8 column (FIG. 3B). This peak correlates with scFv(UCHT-1)-PE38  
9 (>95% pure) as evidenced by mobility at the expected position  
10 (64.5 kD) following SDS-PAGE (FIG.4) and by cross-reaction on  
11 Western blots probed with rabbit anti-PE38 polyclonal antibodies  
12 (not shown in figures).

13  
14       The yield of correctly refolded scFv(UCHT-1)-PE38 recovered  
15 using the above procedure has reached 50 mg/L using the above-  
16 indicated concentrations of DTE and GSSG.

17  
18       The refolding protocol is reproduced in sixteen batches of  
19 material, which are refolded to yield material with very similar  
20 IC<sub>50</sub> values as determined in the MTS assay (Table III).

21  
22       The first eleven batches produce a protein which has a  
23 point mutation which converts serine to arginine at residue 63  
24 in the third framework region of the variable light chain of  
25 UCHT-1. Based on the in vitro results presented on Table III  
26 infra, this mutation appears to have little or no consequence in  
27 terms of the specific in vitro cytotoxicity.

28  
29       Five batches of protein (i.e. batches 12, 13, 14, 15, and  
30 16), in which the point mutation is corrected, are refolded.

1 Due to the high reproducibility in the MTS assay, batches  
2 12 and 13, and batches 14, 15 and 16, are pooled. The pooled  
3 batches are tested for potency in the MTS assay (see Table III)  
4 and then themselves combined to form "Pooled Batches 12-16",  
5 used in the majority of the in vitro studies, and in the in vivo  
6 studies, reported herein. Pooled Batches 10A-12A, also  
7 comprising the corrected material, are similarly obtained and  
8 tested (see Table III).

9  
10 Analysis by non-denaturing PAGE reveals that purified  
11 scFv(UCHT-1)-PE38 exists in solution as a monomer (not shown in  
12 figures). In addition, there appears to be no aggregated  
13 material, as assayed by size exclusion column chromatography  
14 (Sephacryl S200) (Figs. 5A (sample) and 5B (marker)) or by  
15 dynamic light scattering (not shown). Essentially all of the  
16 protein migrates near the position of bovine serum albumin  
17 (66kD).

#### 18 Biological Activity of Immunotoxins.

19

##### 20 (1) MTS assay of scFv(UCHT-1)-PE38.

21 Specific toxicity towards a CD3<sup>+</sup>-expressing human Jurkat T-  
22 cell line is demonstrated using an MTS assay three days after  
23 addition of immunotoxin to cells.  
24

25  
26 In the MTS assay, cell viability is measured by adding MTS,  
27 i.e. (3(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2H-  
28 tetrazolium, inner salt), which is metabolized by viable cells  
29 in the presence of the electron coupling agent, phenazine  
30 methosulfate, to a water-soluble formazan derivative. The  
31 absorbance at 490 nm of the formazan derivative is proportional  
32 to the number of viable cells. The number of viable cells at

1 the time of test compound addition is compared to the number of  
2 viable cells present at 72 hours post-compound addition. The  
3 negative control for non-specific toxicity is the human CD3<sup>+</sup>  
4 Ramos B-cell line.

5

6 The IC<sub>50</sub> and standard deviations of 16 samples of refolded  
7 protein on Jurkat and Ramos are reported on Table III.

1  
2  
3  
4  
5  
6  
7  
8

Table III. Toxicity of different refolded batches on human CD3<sup>+</sup> (Jurkat) or CD3<sup>-</sup> (Ramos) cell lines produced using either a *point variant* of scFv(UCHT-1)-PE38 (Batches 1-11 and Pooled Batches 4-7 and 8-11) or scFv(UCHT-1)-PE38 (Pooled Batches 12-13; 14-16; 12-16; and 10A-12A).

		Jurkat (CD3 <sup>+</sup> )		Ramos (CD3 <sup>-</sup> )	
Batch(es)	Mean IC <sub>50</sub> (ng/ml)	Std. Error of Mean	N	Mean IC <sub>50</sub> (ng/ml)	n
<i>Point variant</i>					
1	1.51	0.37	9	>10 >25	2 4
3	1.03	0.17	5	>25 >250	1 1
4	0.75	0.10	5	>25 >250 >10,000	1 1 1
5	0.57		1	>10,000	1
6	0.18		1	>10,000	1
7	0.27		1	>10,000	1
8	0.18		2	>25	2
9	0.22	0.04	3	>25 >1000	2 1
10	0.21		1	>25	1
11	0.26		1	>25	1
Pooled 4-7	0.26	0.04	5	>25	3
Pooled 8-11	0.27	0.07	3	>25	1
<i>scFv (UCHT-1) - PE38</i>					
Pooled 12-13	0.18		1	>25	1
Pooled 14-16	0.28		1	>25	1
Pooled 12-16	0.63	0.15	16	>25 >10,000	8 4
Pooled 10A-12A	1.3	0.30	7	>100,000	2

1 The scFv(UCHT-1)-PE38 immunotoxin is very potent ( $\approx 10\text{pM}$ ) as  
2 measured by CD3<sup>+</sup> cell killing in the MTS assay. At high  
3 concentrations, the protein reduces the viable cell number below  
4 the starting cell number, and therefore behaves as a cytotoxic  
5 agent.

6  
7 (2) Thermal stability of scFv.

8 The thermal stability of scFv (UCHT-1)-PE38 is measured  
9 using the MTS assay described above. Samples are incubated at  
10 4°C., 25°C. and 37°C. at 100  $\mu\text{g/ml}$  in PBS. As is evident from  
11 Table IV, the material is completely stable at 4°C. and 25°C.  
12 for one month. At 37 °C., there may be a slight increase in the  
13 IC<sub>50</sub> at 21 or 28 days.

14  
15 TABLE IV: Thermal stability of scFv(UCHT-1)-PE38.

16

IC <sub>50</sub> + std. Dev. (ng/ml)			
Time (days)	4 °C	25 °C	37°C
0	2.0 + 0.5	-	-
7	1.6 + 1.1	0.8 + 0.1	1.9 + 0.8
14	1.2 + 0.8	1.4 + 0.4	2.1 + 1.2
21	2.3 + 2.5	1.6 + 0.4	1.6 + 0.9
28	2.4 + 1.0	1.5 + 0.8	3.2 + 1.8

17  
18  
19  
20 (3) Protein synthesis inhibition assay for scFv(UCHT-1)-PE38.  
21 Cells are incubated overnight in the presence or absence of  
22 immunotoxin. The next morning, cells are pulsed for three hours  
23 with <sup>3</sup>H-leucine. The plates are frozen at -80°C for cell lysis,  
24 and then harvested onto a glass filter fibermat using a cell  
25 harvester and extensive water washes. Incorporation into  
26 protein is measured using a Wallac Betaplate reader. Typically,  
27 in the absence of immunotoxin, <sup>3</sup>H-leucine incorporation is 3,000-

1 4,000 cpm; background from label added immediately prior to cell  
2 processing is 400-700 cpm. The standard deviation of triplicate  
3 wells within one plate is generally <10%, and variation of the  
4 mean incorporation between plates is <10%.

5  
6 In Figure 6, protein synthesis inhibition in Jurkat (CD3<sup>+</sup>)  
7 and Ramos (CD3<sup>-</sup>) cells by Pooled Batches 12-16, or Pooled Batches  
8 10A-12A, of scFv(UCHT-1)-PE38 is shown. The plot shows the mean  
9 and standard error of the mean for nine determinations for  
10 pooled Batches 12-16, and for three determinations for Pooled  
11 Batches 10A-12A. The IC<sub>50</sub> of the scFv(UCHT-1)-PE38 in this assay  
12 is 6.7 ± 1.9 ng/ml or 104 ± 29 pM.

13  
14 The curves appear similar from both batches, and the  
15 selectivity for killing is present even at the highest  
16 concentration tested (100 µg/ml). At the higher concentra-  
17 tions, the number of cells is reduced below the starting cell  
18 number.

19  
20 Figure 6 also shows the selectivity of toxicity for the  
21 CD3<sup>+</sup> Jurkat cell line; an IC<sub>50</sub> for killing CD3<sup>-</sup> Ramos cells is not  
22 attained in these experiments even with with 4 or 5-logs higher  
23 concentration of scFv(UCHT-1)-PE38.

24  
25 (4) Human blood Mixed Lymphocyte Reaction (MLR).

26 The ability of the scFv(UCHT-1)-PE38 immunotoxin to prevent  
27 proliferation of alloreactive human peripheral blood mononuclear  
28 cells (PBMC) is measured using a two-way mixed lymphocyte  
29 reaction (MLR). The MLR is a measure of allo-stimulation.  
30 Interference with cell proliferation in the MLR assay is a

1 measure of the potency of an immunosuppressive agent to act upon  
2 intact human blood cells.

3

4 The human MLR is performed according to standard  
5 procedures. PBMC from three different donors (A, B, C) are  
6 isolated on Ficoll from buffy coats with unknown HLA type  
7 (Kantonspital / Basel / Blutspendezentrum). Cells are kept at  $2 \times$   
8  $10^7$  cells/1 ml (90% FCS, 10% DMSO) in cryotubes (Nunc) in liquid  
9 nitrogen until use. To initiate the MLR, the cells are thawed,  
10 washed and counted.

11

12 In each of two experiments ("A" and "B"), 3 individual, 2-  
13 way reactions ( $A \leftrightarrow B$ ,  $A \leftrightarrow C$ ,  $B \leftrightarrow C$ ) are established by mixing cells  
14 from 2 different donors in a ratio of 1:1 by cell number. The  
15 mixed cells (total  $4 \times 10^5$  cells/0.2 ml) are co-cultured in  
16 triplicate for 6 days at  $37^\circ\text{C}$ ., 5%  $\text{CO}_2$ . Cyclosporine A serves as  
17 a positive control.

18

19 Cultures are performed in the presence of increasing  
20 concentrations of immunotoxin (Pooled Batches 12-16) or control.

21

22 Proliferation is determined by  $^3\text{H}$ -TdR uptake (1 mCi/0.2ml)  
23 over the last 16 hours of culture.

24

25 The results are presented on Table V and shown  
26 graphically on Figure 7.

27

Table V. Inhibition of human mixed lymphocyte reactions by scFv(UCHT-1)-PE38 compared to cyclosporine A in two experiments, (I) and (II).

Compound	A<->B	B<->C	B<->C	Mean $\pm$ Std. Dev.
Experiment A				
scFv(UCHT-1)-PE38	0.15 ng/ml	0.13ng/m l	0.05 ng/ml	0.11 $\pm$ 0.053 ng/ml
Cyclosporine A	22.9 nM	17.3 nM	14.4 nM	18.2 $\pm$ 4.32 nM
Experiment B				
scFv(UCHT-1)-PE38	0.036 ng/ml	0.033 ng/ml	0.036 ng/ml	0.035 $\pm$ 0.002 ng/ml
Cyclosporine A	2.6 nM	1.6 nM	2.6 nM	2.27 $\pm$ 0.58 nM

The potency of scFv(UCHT-1)-PE38 in preventing proliferation of human blood PBMC in an in vitro mixed lymphocyte reaction (MLR) in the above two experiments is determined to be  $0.11 \pm 0.053$  ng/ml and  $0.035 \pm 0.002$  ng/ml, resulting in a global  $IC_{50}$  of  $0.072 \pm 0.053$  ng/ml (1.12 pM).

The data demonstrate that scFv(UCHT-1)-PE38 efficiently suppresses allo-specific T cell activation in human MLR.

(5) Inhibition of human CD3 $\epsilon$  transgenic murine splenocyte Concanvalin A- stimulated proliferation by scFv(UCHT-1)-PE38.

Human CD3 $\epsilon$  transgenic mice: A strain of human CD3 $\epsilon$  transgenic mice is obtained from C. Terhorst (Beth Israel



1 Deaconess Medical Center). The phenotype of transgenic mice  
2 expressing high and low copy numbers of human CD3 $\epsilon$  is described  
3 by Wang et al. (1994) *PNAS* 91: 9402. Mice which express high  
4 copy numbers of the transgenic human CD3E gene have no T or NK  
5 cells even when heterozygous, and thus have a knockout  
6 phenotype. The tg $\epsilon$ 600 strain reportedly has ~3 copies of the  
7 human CD3 $\epsilon$  transgene integrated chromosomally at an unknown  
8 location. Homozygous, low-copy number transgenic mice such as  
9 tg $\epsilon$ 600 mice express only a limited number of T cells. In  
10 contrast, when heterozygous for tge600, mice have near normal  
11 numbers of T cells most of which express both human and murine  
12 CD3 $\epsilon$ .

13  
14 The genetic background of these mice is mixed; the  
15 transgene being introduced by pronuclear injection of F2 embryos  
16 from a CBA by C57BL/6 cross, and therefore, siblings are  
17 genetically different.

18  
19 The transgenic mice homozygous for human CD3 $\epsilon$  are bred at  
20 Charles River Laboratories with C57BL/6 wildtype mice to  
21 generate heterozygous mice.

22  
23 The animals are maintained as homozygotes for the trans-  
24 gene and used as heterozygotes after back-crossing to C57BL/6.

25  
26 Animals heterozygous for the tg $\epsilon$ 600 insertion are used for  
27 testing in vitro sensitivity to scFv(UCHT-1)-PE38 and in vivo  
28 depletion caused by scFv(UCHT-1)-PE38 after intravenous or  
29 intraperitoneal administration. Pooled Batch 12-16 was used for  
30 these experiments. For the in vitro work, F1 progeny of a CBA x  
31 C57BL/6 cross are used as control animals. In the in vivo

1 experiments, untreated heterozygous tge600 mice serve as a  
2 control group.

3  
4 The ability of scFv(UCHT-1)-PE38 to inhibit in vitro  
5 proliferation of splenocytes from transgenic mice expressing  
6 human CD3 $\epsilon$  is assessed by Concanavalin A-induced proliferation  
7 (Fig. 8) as well as a one-way mixed lymphocyte reaction (Fig.  
8 9).

9  
10 The spleens are disrupted, passed through a nylon filter  
11 (0.45  $\mu$ m), and gently pipetted with a 1 ml syringe to generate a  
12 single cell suspension. Red blood cells are lysed using ACK  
13 buffer (0.15 M ammonium chloride, 1 mM potassium carbonate, 0.1  
14 mM EDTA), and the resulting suspension washed three times into  
15 RPMI-1640 supplemented with 5% FBS. Concanavalin A (Sigma) is  
16 added to the wells at 5 ug/ml. The plates are incubated for  
17 three days at 37 °C in 5% CO<sub>2</sub>. On the third day, 1 uCi/well of  
18 <sup>3</sup>H-thymidine is added. After 24 hours the cells are harvested  
19 onto glass fiber filters, and the <sup>3</sup>H-thymidine incorporation  
20 measured using a Wallac beta plate reader.

21  
22 As shown in Figure 8, addition of scFv(UCHT-1)-PE38 blocks  
23 Con A (5 ug/ml)- induced proliferation of human CD33 $\epsilon$  transgenic  
24 ("HuCD3 $\epsilon$ Tg") splenocytes, but not proliferation of non-  
25 transgenic, B6CBAF1 ("NonTg") splenocytes. Dose-dependent  
26 inhibition of the cells from the transgenic mice is observed  
27 with a calculated IC<sub>50</sub> of 0.6 ng/ml. This is in good agreement  
28 with cytotoxicity against Jurkat cells (0.63  $\pm$  0.15 ng/ml). At  
29 high concentrations, >100% inhibition is observed (i.e. less  
30 proliferation than observed in the absence of ConA), suggesting  
31 that all ConA-responsive splenocytes are sensitive to scFv(UCHT-  
32 1)-PE38. The line labelled "No ConA" represents the

1 proliferative response in the absence of ConA, due to media  
2 alone.

3  
4 (6) Inhibition of proliferation of human CD3 $\epsilon$  transgenic murine  
5 splenocytes by scFv(UCHT-1)-PE38 in one-way MLR.

6 The ability of scFv(UCHT-1)-PE38 to inhibit human CD3 $\epsilon$   
7 splenocyte T cell proliferation in vitro is assessed using a  
8 one-way mixed lymphocyte reaction. In a one-way MLR,  
9 proliferation is due to direct recognition of allo-MHC II by  
10 allo-reactive huCD3 $\epsilon$  transgenic murine splenocytes. Not all T  
11 cells are allo-reactive, resulting in a smaller percentage of  
12 responding transgenic splenocytes, consistent with the reduced  
13 signal to noise of the assay and the increased variability  
14 between experiments.

15  
16 HuCD3 $\epsilon$  transgenic splenocytes ("CD3Tg cells") are prepared  
17 as in section 5 above. Spleen cells of non-transgenic B6CBAF1  
18 mice ("NonTg cells") are used as a control.

19  
20 A single cell suspension of Balb/C splenocytes prepared as  
21 in section 5 above is treated with mitomycin C (30  $\mu$ g/ml) for 20  
22 min at 37 °C, and washed into MLR media.

23  
24 The mitomycin C-treated BALB/c stimulator cells are added  
25 to flat-well Corning 96-well plates at  $4 \times 10^5$  cells/ml.  
26 Splenocytes from the transgenic mice are added to the wells at  
27  $2 \times 10^5$  cells/ml, and the plates incubated for three days at 37 °C  
28 in 5% CO<sub>2</sub>. On the third day, 1  $\mu$ Ci/well of <sup>3</sup>H-thymidine is  
29 added. After 16 hours, the cells are harvested onto glass fiber  
30 filters, and <sup>3</sup>H-thymidine incorporation measured using a Wallac  
31 beta plate reader.

32

1 As shown in Figures 9A and 9B, the scFv(UCHT1)-PE38  
2 immunotoxin inhibits the allogeneic MLR response in cultures  
3 containing huCD3 $\epsilon$  Tg splenocytes, but not non-transgenic control  
4 splenocytes. Dose-dependent inhibition of the cells from the  
5 transgenic mice is observed, with a calculated IC<sub>50</sub> of 0.6 ng/ml.  
6 At high concentrations, >100% inhibition is observed, suggesting  
7 that all allo-reactive huCD3 $\epsilon$  T cells are sensitive to scFv(UCHT-  
8 1)-PE38. The MLR response between non-transgenic B6CBAF1 spleen  
9 cells and mitomycin C treated Balb/C (APC) splenocytes is not  
10 inhibited by scFv(UCHT-1)-PE38 (Figure 9A).

11  
12 Accordingly, the immunotoxin is found to inhibit a MLR  
13 response of huCD3 $\epsilon$  transgenic splenic (T-cells) cells stimulated  
14 by fully allogeneic mitomycin C-treated BALB/C splenic (APC)  
15 cells, in a dose-dependent manner.

16  
17 The potency of the immunotoxin in this assay is ~0.9 ng/ml,  
18 i.e., ~14 pM.

19  
20 (7) Jurkat hollow fiber implant model

21 Eight hollow fibers are implanted into a single nude mouse:  
22 four are placed intraperitoneally, and another four are placed  
23 subcutaneously. two of the four hollow fibers in each location  
24 contain CD3<sup>+</sup> Jurkat cells; one of the four fibers in each  
25 location contains LS174T colon carcinoma cells; and one contains  
26 MDA-MB-435S breast carcinoma cells. Six animals comprise a  
27 group.

28  
29 It is noted that the material used for these studies  
30 contains a point mutation from T to G at nucleotide 195 of Seq.  
31 ID NO:2 that changes serine (UCHT-1) to arginine (mutant) at  
32 residue 65 of SEQ. ID NO:1 (i.e. in the third framework region  
33 of the variable light chain). The efficacy of this material in

1 the 3-day MTS assay is equivalent to that of scFv(UCHT-1)-PE38  
2 with no mutation (Table III).  
3

4 Figure 10 depicts relative cell growth of Jurkat cells in  
5 hollow fibers implanted in the peritoneal cavity in nude mice,  
6 following intraperitoneal administration (150  $\mu$ L in saline  
7 vehicle per mouse) of scFv(UCHT-1)-PE38 at a dose level of 1 $\mu$   
8 g/mouse twice daily or 5  $\mu$ g/mouse twice daily from days 3-6.  
9 The fiber is retrieved on day 10.  
10

11 Also in this model, approximately 75% inhibition of Jurkat  
12 cell growth in intraperitoneally implanted hollow fibers is seen  
13 using 1  $\mu$ g/mouse dosed i.p. (twice daily for 4 days) or using 3  
14  $\mu$ g/mouse dosed i.v. (twice daily for 4 days).  
15

16 The immunotoxin is shown to have systemic in vivo efficacy  
17 in killing a human T-cell line implanted in nude mice after i.p.  
18 or i.v. administration, and the growth inhibition observed is  
19 specific for CD3<sup>+</sup> cells.  
20

#### 21 (8) T-cell depletion in human CD3 $\epsilon$ transgenic mice.

22 Tg $\epsilon$ 600/C57BL6 heterozygous mice described as above are  
23 treated with 4  $\mu$ g/mouse of immunotoxin (Pooled batches 12-16)  
24 twice daily for four days. One day following the final  
25 treatment, lymph nodes (LN) and spleens are removed, and single  
26 cell suspensions are prepared from individual mice.  
27

28 The percentage of CD3-positive cells is assessed by two-  
29 color FACS analysis performed on single cell suspensions using  
30 FITC-anti huCD3 $\epsilon$  antibodies (to measure expression of human CD3 $\epsilon$

1 and phycoerythrin (PE) conjugated-anti mCD3e antibodies (500A2-  
2 PE) (to measure expression of mouse CD3). The number of T cells  
3 in each organ is determined by multiplying the number of total  
4 cells recovered from the organ by the percentage of CD3-positive  
5 cells.

6  
7 Figures 11A,B and C and Figures 12A, B and C show  
8 representative FACS analyses of the spleen (Fig. 11), and the  
9 lymph node (Fig. 12) from treated and untreated animals. Each  
10 figure shows three plots as follows: (A) cells from untreated  
11 mice stained with control antibodies of identical isotype to the  
12 test antibodies; (B) cells from untreated mice double-stained  
13 with anti-human and anti-mouse CD3 MAb's; and (C) cells from mice  
14 treated with scFv(UCHT-1)-PE38 double-stained with anti-human and  
15 anti-mouse CD3 MAb's.

16  
17 Figure 11A shows that non-specific staining of cells by  
18 isotype matched control antibodies is low. No difference in non-  
19 specific staining is seen between treated or untreated mice  
20 (data not shown).

21  
22 Figure 11B shows that ~20% of the total cells in the spleen  
23 in an untreated transgenic animal are positive for both mCD3 and  
24 huCD3 (upper right quadrant). A small percentage of cells  
25 express mouse CD3, but do not express human CD3 (3.5%; upper  
26 left quadrant).

27  
28 Figure 11C shows that systemic treatment with scFv(UCHT-1)-  
29 PE38 reduces the percentage of cells that express both huCD3 and  
30 mCD3 from about 20% to 2%.

31

1       The results of FACS analyses of lymph nodes (LN) from  
2 treated and untreated transgenic mice shown in Figure 12 are  
3 similar to the results seen in the FACS analysis of spleen cells  
4 from the transgenic mice. That is, non-specific staining of  
5 cells by isotype matched control antibodies is low (Figure 12A).  
6 In an untreated transgenic mouse, ~53% of the total cells in the  
7 LN are positive for both mCD3 and huCD3 (upper right quadrant,  
8 Figure 12B). A small percentage of cells express mouse CD3, but  
9 do not express human CD3 (2.8%; upper left quadrant). After  
10 intravenous administration of scFv(UCHT-1)-PE38 (4 µg/animal)  
11 twice daily for four days, the percentage of double positive LN  
12 cells that express huCD3 and mCD3 is reduced from ~53% to 12%  
13 (Figure 12C).

14  
15       The effect of different dosing regimens on the percentage  
16 and number of cells double positive for both mouse and human CD3  
17 is shown for the three tested tissues in Figures 13A and B and  
18 14A and B`. Results are similar for both spleen (Figure 13)  
19 and lymph node (Figure 14). scFv(UCHT-1)-PE38 causes  
20 statistically significant depletion of double positive T-cells  
21 when administered either i.v. or i.p. in a twice a day dosing  
22 regimen. In addition, dose-dependent depletion is observed in  
23 both tissues after systemic administration.

24  
25       Summarizing the data generated, 4 µg/mouse i.v. or 5 µ  
26 g/mouse i.p. for 4 days b.i.d. result in 86% and 95% depletion in  
27 the number of splenic huCD3 T cells recovered. Statistic-ally  
28 significant reduction of spleen cell number is seen with 0.3 µ  
29 g/mouse i.v. b.i.d x 4 days and with 1 µg/mouse i.v. b.i.d. when  
30 the percentage of huCD3 positive cells is considered. Thus the  
31 lowest effective dose appears to be 1 µg b.i.d. x 4 days for  
32 splenic depletion.

1        For the lymph node, treatment with 4 µg/mouse i.v. or 5 µ  
2 g/mouse i.p. for 4 days b.i.d. results in 97% and 92% depletion  
3 in the number of huCD3 T cells recovered. Statistically  
4 significant reduction of lymph node cell number is seen in mice  
5 treated with 3 µg/mouse i.v. b.i.d x 4 days and with 1 µg/mouse  
6 i.v. b.i.d. x 4 days when the percentage of huCD3 positive cells  
7 in lymph node is considered. Thus, the lowest effective dose  
8 appears to be 1µg b.i.d. x 4 days for lymph node depletion.



# SEQUENCE LISTING

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 . Lake, Philip  
 Wright, Richard M.

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<223> Description of Artificial Sequence: PE peptide

<400> 6  
Arg Glu Asp Leu Lys  
1 5

<210> 7  
<211> 4  
<212> PRT  
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<220>  
<223> Description of Artificial Sequence: PE peptide

<400> 7  
Arg Glu Asp Leu  
1

<210> 8  
<211> 4  
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<220>  
<223> Description of Artificial Sequence: PE peptide

<400> 8



Lys Asp Glu Leu

1

<210> 9

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: peptide  
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<400> 9

Lys Ala Ser Gly Gly

1

5

<210> 10

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: peptide linker

<400> 10

Gly Gly Gly Gly Ser

1

5

<210> 11

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer IM-34A

<400> 11

gcggatccga catccagatg acccagacca cc

32

<210> 12

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer IM-34B

<400> 12

cctctagaag cccgtttgat ttccagcttg gt

32

<210> 13

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer IM-34C

<400> 13

ccaagctttc atgaggagac ggtgaccgtg gtccc

35

<210> 14

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer IM-61

<400> 14

ccgtcgacga ggtgcagctc cagcagtct

29

<210> 15

<211> 42

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Oligo IM24A

<400> 15

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42

<210> 16

<211> 42

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Primer IM-24B

<400> 16

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42

<210> 17  
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<220>  
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<400> 17  
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23

<210> 18  
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<220>  
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27

<210> 19  
<211> 27  
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<220>  
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<400> 19  
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27

<210> 20  
<211> 21  
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<220>  
<223> Description of Artificial Sequence: Primer VH4

<400> 20  
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21

<210> 21  
<211> 66  
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<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer VL6

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cttggt 66

<210> 22

<211> 57

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Primer VL8

<400> 22

tcagggtccag actgctggag ctgcacctca gatccgccac cgctgcctcc acctgat 57

ccggtccag actgctggag ctgcacctca gatccgccac cgctgcctcc acctgat